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Characterization of the Induction of Aluminum Resistance in *Phaseolus vulgaris* L.

by

Nicole Paulette Bisson



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science

in

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of the Induction of Aluminum resistance in *Phaseolus vulgaris* L.** submitted by **Nicole Paulette Bisson** in partial fulfillment of the requirements for the degree of **Master of Science in Plant Biology**.

For Daniel

ABSTRACT

Differential resistance to aluminum (Al) was exhibited between snapbean (*Phaseolus vulgaris* L.) cultivars Dade (resistant) and Romano (sensitive) over the long-term (16 d) when grown in a high volume system. However, root elongation was inhibited in both cultivars during the first 24 h of Al exposure. Subsequently, root elongation of cv. Dade recovered to control levels, suggesting that resistance to Al was induced in Dade.

Differential citrate exudation was measured in both cultivars after 24 h of exposure to Al in a low volume system. Increased exudation of organic anions such as citrate may play a role in Al detoxification in Dade, but this could not be confirmed as depletion of Al occurred in the low volume system. The induction of Al resistance in Dade was further characterized in the high volume system. A clear coincidence in time between the recovery from Al-induced inhibition of root elongation, the disappearance of cortical cell injury, and hematoxylin staining patterns provides evidence of induced Al resistance in cv. Dade.

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LIST OF ABBREVIATIONS

Al.....aluminum

βbeta

$^{\circ}\text{C}$degrees Celcius

cv.....cultivar

DIC.....differential interference microscopy

H_2Owater

ID; OD.....inside diameter; outside diameter

N.....normal

NADH.....reduced nicotinamide adenine dinucleotide

nm; μm ; mm; cmnanometer; micrometer; millimeter; centimeter

nmol; μmolnanomole; micromole

s; min; h; d.....second; minute; hour; day

SE.....standard error

U.....units

μg ; mg; gmicrogram; milligram; gram

μL ; mL ; Lmicroliter; milliliter; liter

μM ; mM ; Mmicromolar; millimolar; molar

v/v ; w/vvolume per volume; weight per volume

$\times g$multiplied by the standard acceleration of
gravity

$\%$percent

$<$; $>$less than; greater than

$\leq$; $\geq$less than or equal to; greater than or equal to

1. INTRODUCTION

Soil acidity is a worldwide problem that has considerable agronomic and economic repercussions. It affects up to 40% of agricultural soils (Haug, 1984) and is associated with a reduction in soil productivity and plant nutrient availability. Generally soils with a pH of less than 5.5 are classified as acidic (Marschner, 1995). Sources of soil acidity include high organic matter content, the amount and nature of clay colloids present, presence of hydrous oxides of aluminum (Al) and iron (Fe), high levels of exchangeable Al^{3+} and soluble salts, leaching of basic cations, and elevated carbon dioxide (CO_2) in the soil atmosphere. Current agronomic practices also contribute to reductions in soil pH. The use of fertilizers, particularly ammoniacal (NH_4^+) sources that release protons (H^+) under conditions favoring nitrification, crop removal, and decomposition of crop litter all encourage soil acidification. In addition, pollutants such as sulfur dioxide (SO_2) and nitrogen dioxide (NO_2) released as industrial emissions exacerbate acidity. These pollutants react with water in the atmosphere to form sulfuric and nitric acids and enter the soil as precipitation (Tisdale *et al.*, 1993).

Aluminum is ubiquitous in mineral soils and Al toxicity is the most important factor limiting crop growth in acid soils. As pH decreases, Al is solubilized and becomes biologically available and rhizotoxic. The resulting inhibition in root growth affects nutrient and water uptake, transport, and subsequently, shoot growth. Liming soils is a common agricultural practice used to manage soil acidity and Al phytotoxicity.

Unfortunately, application of lime is not always economically feasible and can have

detrimental effects on soils after prolonged use. An alternative recourse is the use of genetic and molecular manipulations to develop crops able to grow in the presence of Al. For these strategies to be successful, scientists must first gain an understanding of the physiological and biochemical basis of Al toxicity and the mechanisms that allow certain plants to grow in acidic, Al-toxic soils.

1.1. Aluminum Toxicity

1.1.1. Aluminum speciation in aqueous systems

Aluminum is the most abundant metal in the earth's crust, representing approximately 8% of total metals (Martin, 1988; Marshner, 1995). Aluminum forms various species in aqueous systems, making its chemistry complex. In acidic solutions ($\text{pH} < 5.0$), Al is released from soil minerals to the soil solution as hexaaquaaluminum ($\text{Al}^{3+} \cdot 6\text{H}_2\text{O}$ or Al^{3+}). As solutions become less acidic ($5.0 < \text{pH} < 6.2$), $\text{Al}^{3+} \cdot 6\text{H}_2\text{O}$ undergoes deprotonation to form mononuclear hydrolysis products, $\text{Al}(\text{OH})^{2+} \cdot 5\text{H}_2\text{O}$ and $\text{Al}(\text{OH})_2^+ \cdot 4\text{H}_2\text{O}$. Gibbsite ($\text{Al}(\text{OH})_3 \cdot 3\text{H}_2\text{O}$), an insoluble mononuclear product, is predominant at a neutral pH, while aluminate ($\text{Al}(\text{OH})_4^- \cdot 2\text{H}_2\text{O}$) dominates under alkaline conditions ($\text{pH} > 6.2$). Polynuclear species may also appear under these conditions (Martin, 1988; Matsumoto, 2000). Aluminum has a strong binding affinity for oxygen donor ligands, and formation of Al complexes occurs when divalent or trivalent ions react with organic (e.g. citrate, malate) and inorganic ligands (e.g. fluoride, sulfate, phosphate, silicate) (Uren, 1989; Kinraide, 1991; Yokel, 2002).

1.1.2. The phytotoxic form of aluminum

The high level of reactivity of Al in aqueous systems makes it challenging to identify specific rhizotoxic Al species. The presence of ligands, and the pH, ionic strength, and age of experimental solutions affect Al speciation, making it difficult to determine what particular Al species is in solution. In addition, equilibrium constants used to calculate the activity of individual Al species in solution are subject to error (Parker *et al.*, 1989; Kinraide, 1991). Polynuclear hydroxy-Al species can arise with pH changes or when Al concentrations rise in solutions. Often the presence of highly toxic polynuclear triskaidekaaluminum ($\text{AlO}_4\text{Al}_{12}(\text{OH})_{24}^{7+} \cdot 12\text{H}_2\text{O}$ or Al_{13}) is undetected or unpredicted, and can lead to wrongful attribution of toxicity to other Al species (Parker *et al.*, 1988; Kinraide and Parker, 1990; Kinraide, 1991).

Interactions between Al^{3+} and H^+ can also make it difficult to determine whether an Al species is actually toxic. It is hypothesized that H^+ occupies potential Al^{3+} binding sites on the root surface, reducing Al^{3+} toxicity. Conversely, at a solution $\text{pH} < 4.0$, Al^{3+} may alleviate H^+ toxicity, leading to the erroneous conclusion that Al species formed at higher pH are more phytotoxic than those formed at lower pH. Furthermore, the identity and activity of Al species at root cell surfaces is unclear because the chemistry of root free spaces is uncertain (Kinraide, 1991). Nonetheless, scientists generally agree that rhizotoxicity is associated with the appearance of Al_{13} and Al^{3+} . Polynuclear hydroxy-Al species may also be toxic under basic conditions (Parker *et al.*, 1988). Correlative evidence suggests that AlF_2^{2+} and AlF_2^+ are toxic to wheat at high levels (Kinraide, 1997). $\text{Al}(\text{OH})_2^{2+}$ and $\text{Al}(\text{OH})_2^+$ are toxic to certain dicot plants, but only under conditions

representing Al activities generally unattainable in soil. Aluminate ($\text{Al}(\text{OH})_4^-$), AlSO_4^+ , $\text{Al}(\text{SO}_4)_2^-$, and organic complexes are considered non-rhizotoxic (Kinraide, 1997).

1.1.3. Aluminum toxicity in plants

Characteristic symptoms of Al toxicity include an inhibition of growth of the main root axis and lateral roots. Roots are thick, stubby, brown, brittle, and lack fine branching, giving them a coralloid appearance (Clarkson, 1965; Fleming and Foy, 1968; Keser *et al.*, 1975, 1977; Foy *et al.* 1978; Foy, 1984). The root apex appears to be the primary site of Al toxicity. Studies with maize show that a toxic response occurs only when the terminal 1 to 3 mm of the root, specifically the distal part of the transition zone of the root apex, is exposed to Al (Ryan *et al.*, 1993; Sivaguru and Horst, 1998; Kollmeier *et al.*, 2000).

At the cellular level, Al interferes with cell elongation (Marienfeld and Stelzer, 1993; Horst, 1995; Sasaki *et al.*, 1997) and cell division (Clarkson, 1965; Naidoo *et al.*, 1978, Bennet *et al.*, 1987; Tepper *et al.*, 1989; Vázquez *et al.*, 1999; Silva *et al.*, 2000). Various reports suggest that Al is associated with changes in the cell wall (Wheeler *et al.*, 1992; Le Van *et al.*, 1994; Gunsé *et al.*, 1997; Vázquez *et al.*, 1999; Sasaki *et al.*, 1997; Chang *et al.*, 1999), damage to the plasma membrane (Taylor, 1988; Miyasaka *et al.*, 1989; Wagatsuma *et al.*, 1995), lipid peroxidation (Cakmak and Horst, 1991; Horst *et al.*, 1992; Basu *et al.*, 2001; Ezaki *et al.*, 2001), and increased vacuolation (Bennet *et al.*, 1985a, 1985b; Hecht-Buchholz *et al.*, 1990; Johnson and Bennet, 1991; Wheeler *et al.*, 1992). Studies also show that Al associates with nuclei (Matsumoto, 1991; Silva *et al.*, 2000), has an effect on the cytoskeleton (Sasaki *et al.*, 1997; Blancaflor, *et al.*, 1998; Sivaguru *et*

al., 1999a, 1999b), and on enzyme-mediated reactions (de Lima and Copeland, 1994).

There is some controversy as to which of these are direct effects of Al toxicity, and scientists are uncertain of the primary mechanism of Al toxicity in roots. Nonetheless, all of these symptoms are important as each contributes to the overall toxic response.

1.2. Aluminum Resistance

1.2.1. Differential resistance to aluminum

Not all plants respond to Al similarly. Several cultivars of important crop species exhibit phenotypic variability when grown in the presence of Al. Differential resistance to Al exists in a variety of species, including snapbean, wheat, and maize. For example, the snapbean cultivar, Dade, is 2 fold more resistant than cultivar, Romano (Foy *et al.*, 1972). Resistant varieties of maize are also 2 to 3 times more resistant to Al than sensitive varieties (Pellet *et al.*, 1995). Likewise, Al-resistant lines of wheat withstand up to 10 times more Al than sensitive lines (Delhaize and Ryan, 1995).

1.2.2. Mechanisms of aluminum resistance

Hypotheses for Al resistance mechanisms in plants are generally separated into two categories based on the site of Al detoxification. Plants resist Al by using external and/or internal mechanisms to avoid or tolerate Al stress. External mechanisms function by detoxifying or immobilizing Al in the apoplasm. These mechanisms protect sensitive sites in the apoplasm and/or limit the rate of uptake across the plasma membrane. External mechanisms could include immobilization of Al at the cell wall, selective permeability at the plasma membrane, low cell wall cation exchange capacity, plant-

induced changes in rhizosphere, changes in apoplasmic pH, Al efflux, and the exudation of phosphate or chelating agents (Taylor, 1991, 1995). Internal resistance mechanisms involve Al detoxification or immobilization in the symplasm. These mechanisms are important where external mechanisms are absent or not fully effective. Potential mechanisms include chelation of Al in the cytosol by organic anions or proteins, compartmentation in the Al insensitive vacuole, the evolution of Al-resistant enzymes, and elevated enzyme activity (Taylor, 1991, 1995).

1.2.3. Organic acids in plants

A large body of evidence supports the role of organic acids/anions in mediating both external and internal mechanisms of Al resistance. Organic acids are low molecular weight compounds that possess one or more carboxyl groups. These molecules carry various negative charges, depending on the number of carboxylic groups, dissociation properties, and solution pH (Jones, 1998). Some organic acids act as intermediates in the tricarboxylic acid (TCA) cycle and are involved in energy production and biosynthetic reactions. Others occur in cells to balance excess cation charges, regulate cytosolic pH, and maintain osmotic potential (Jones, 1998; Ryan *et al.*, 2001). Most organic acids exist as fully dissociated anions in the near-neutral pH of the cytosol. In the anionic form, their negative charge allows them to form stable complexes with metal cations in solution. The nutritional status, age, and the type of carbon fixation (CAM, C3, C4) exhibited by a particular plant can affect its organic acid/anion content. Roots contain many organic anions, including lactate, acetate, oxalate, succinate, fumarate, malate, citrate, isocitrate, and aconitate (Jones, 1998).

1.2.4. The role of organic anions in aluminum detoxification

Several organic anions readily form stable complexes with Al and appear to play an important role in mediating both external and internal mechanisms of Al resistance. Upon binding, the positive charge of Al is neutralized and the activity of the free Al in solution is reduced, protecting sensitive extracellular sites (Kinraide, 1991). Formation of a complex may also protect sensitive intracellular sites by preventing or reducing Al absorption across the plasma membrane through size and/or charge modification or immobilization. If these mechanisms are absent or ineffective, chelation of Al in the cytosol by organic anions may limit phytotoxicity at the cellular level. At present, the stability of these complexes in the cytosol is uncertain, as the solubility of free Al^{3+} is limited at near-neutral pH (Taylor, 1991).

Scientists have extensively investigated the effect of organic anions on Al toxicity in plants. Hue *et al.* (1986) correlated amelioration of Al toxicity with the log of the stability constants of various Al-organic anion complexes. Organic anions were divided into three groups according to their ability to detoxify Al^{3+} . Strong detoxifiers included citrate, oxalate, and tartarate, with citrate being the strongest. Malate, malonate, and salicylate were moderate detoxifiers, while succinate, lactate, formate, acetate, and phthalate were classified as weak detoxifiers. The arrangement of hydroxyl (OH) and carboxyl (COOH) groups on an organic acid's main carbon chain was also correlated with detoxification ability. Strong detoxifiers have two pairs of OH and/or COOH groups connected to adjacent carbons, or two COOH groups directly bound to each other. This favors formation of a stable 5 or 6 bond ring structure with Al^{3+} (Hue *et al.*, 1986). In another

study, Bartlett and Riego (1972) showed that the addition of organic ligands (citrate, EDTA, soil organic matter extract) to Al solutions alleviated Al toxicity in maize. A number of subsequent experiments have also shown similar results (Delhaize *et al.*, 1993b; Basu *et al.*, 1994; Ryan *et al.*, 1995b; Pellet *et al.*, 1996; Ginting *et al.*, 1998; Ma and Miyasaka, 1998; Yang and Zhang, 1998; Luo *et al.*, 1999; Saber *et al.*, 1999).

1.2.5. Aluminum-induced exudation of organic anions

Numerous studies have correlated the exudation of organic anions in the rhizosphere and differential resistance to Al. Miyasaka *et al.* (1991) documented that an Al-resistant cultivar of snapbean, Dade, exuded 10 times more citrate than an Al-sensitive cultivar, Romano, in the presence of Al. Similarly, Al-resistant lines of wheat excreted 5-10 fold more malate than sensitive lines (Delhaize and Ryan, 1993b). Exudation of organic anions has been documented in a variety of Al-resistant species, including malate in wheat (Delhaize *et al.*, 1993b; Basu *et al.*, 1994; Ryan *et al.*, 1995a, 1995b; Pellet *et al.*, 1996; Li *et al.*, 2000; Osawa and Matsumoto, 2001), citrate in maize (Pellet *et al.*, 1995; Gaume *et al.*, 2001; Piñeros *et al.*, 2002) and snapbean (Miyasaka *et al.*, 1991; Mugai *et al.*, 2000), and oxalate in buckwheat (Zheng *et al.*, 1998) and taro (Ma and Miyasaka, 1998). In addition, species such as maize (Jorge and Arruda, 1997; Kollmeier *et al.*, 2001), barley (Gallardo *et al.*, 1999), and triticale (Ma *et al.*, 2000) exude several organic anions in response to Al. Studies investigating the primary site of organic anion release are contradictory. Pellet *et al.* (1995) demonstrated that citrate exudation in maize is confined to the root apex. Likewise, Delhaize *et al.* (1993b) measured malate release in wheat primarily from the terminal 3 to 5 mm of the root. On the other hand, Zheng *et al.*

(1998) measured oxalate secretion in buckwheat in the region 0 to 10 mm from the root tip, and Piñeros *et al.* (2002) measured substantial citrate exudation in maize as far as 5 cm beyond the root cap.

1.2.6. Organic anion efflux from roots

Mechanisms regulating organic anion efflux from roots are not well known. The plasma membrane ATPase creates an electrical potential gradient (negative inside) across the plasma membrane by ATP-driven export of H^+ to the apoplasm (Marschner, 1995). In addition, the concentration of organic anions is typically 1000 fold greater in the cytosol than in the soil solution (Jones, 1998). This results in a large electrochemical potential gradient across the plasma membrane, favoring efflux of anions from cells. Scientists hypothesize that anion channels embedded in the lipid bilayer facilitate efflux of anions from the cytoplasm to the soil solution. Evidence supporting this hypothesis comes from studies with inhibitors and measurements of ion currents in protoplasts using the patch-clamp technique. Several groups have applied various anion channel antagonists to roots of different plant species and observed subsequent inhibition of organic anion exudation in response to Al (Ryan *et al.*, 1995a; Papernik and Kochian, 1997; Zheng *et al.*, 1998; Li *et al.*, 2000). Unfortunately, anion channel inhibitors have uncertain specificities and do not always have a consistent effect on organic anion release.

Osawa and Matsumoto (2001) demonstrated that application of a protein kinase inhibitor blocked Al-activated malate efflux in an Al-resistant cultivar of wheat. This resulted in increased Al accumulation in root apices and inhibited root growth. They suggested that

protein phosphorylation may be related to gating of anion channels, or involved in the signal transduction pathway leading to Al-stimulated malate efflux through anion channels. Anion channels activated by Al^{3+} have been observed in wheat and maize root cell protoplasts (Ryan *et al.*, 1997; Kollmeier *et al.*, 2001; Piñeros and Kochian, 2001; Zhang *et al.*, 2001; Piñeros *et al.*, 2002). Several of these channels are permeable to specific organic anions. In addition, patterns of Al^{3+} activation reflect those of organic anion efflux from whole plants. The existence of Al-gated anion channels in the plasma membrane has yet to be confirmed at the molecular level, and more evidence regarding the perception and transduction of the Al signal is required.

Other possible mechanisms of transport that have not been extensively explored include trans-membrane diffusion of lipophilic organic anions and exocytosis (Ryan *et al.*, 2001). Total organic acid/anion concentrations in the root can be measured, but concentrations vary at different sites along the root (Jones and Darrah, 1995). In addition, cellular compartmentation of anions makes it difficult to relate these concentrations to efflux. Several groups have reported that root exudation does not reflect the composition of internal pools of organic anions (Delhaize *et al.*, 1993b; Pellet *et al.*, 1995; Ryan *et al.*, 1995a; Zheng *et al.*, 1998; Gaume *et al.*, 2001). Large amounts of organic acids are stored in the vacuole, but it is the electrochemical gradient between the cytosol and soil solution that ultimately determines efflux rates (Jones, 1998).

1.2.7. Patterns of aluminum-stimulated organic anion efflux

Scientists have described two patterns of organic anion efflux from Al-resistant plants in response to Al stress. The first pattern entails an immediate release of organic anions, while in the second there is a time lag between Al exposure and exudation (Ma, 2000). Researchers hypothesize that the first pattern is characteristic of plants that possess the metabolic machinery required to produce and transport organic anions. Aluminum activates pre-existing or constitutively expressed biosynthetic and/or transport mechanisms, resulting in an immediate response (Ma, 1997, 2000; Ma *et al.*, 2001; Ryan *et al.*, 2001). This pattern is described in certain wheat varieties that exude malate (Delhaize *et al.*, 1993b; Ryan *et al.*, 1995a; Li *et al.*, 2000; Osawa and Matsumoto, 2001), a tobacco variety that releases citrate (Delhaize *et al.*, 2001), and buckwheat cultivars exuding oxalate in response to Al (Ma *et al.*, 1997b; Zheng *et al.*, 1998). Some wheat and buckwheat cultivars release organic anions as quickly as 5 to 30 minutes after Al exposure (Delhaize *et al.*, 1993b; Ryan *et al.*, 1995a; Ma *et al.*, 1997b; Zheng *et al.*, 1998; Li *et al.*, 2000; Osawa and Matsumoto, 2001).

Scientists postulate that the second pattern involves inducible processes, and reflects the need for *de novo* synthesis of biosynthetic or transport proteins before organic acids are released (Ma, 2000; Ma *et al.*, 2001; Ryan *et al.*, 2001). Maize and soybean cultivars show delayed exudation of citrate (Pellet *et al.*, 1995; Yang *et al.*, 2000), as do Al-resistant triticale (Ma *et al.*, 2000), *Cassia tora* (Ma *et al.*, 1997a), and rye (Li *et al.*, 2000, 2002). In these plants, the onset of citrate exudation occurs from 4 to 48 hours after Al exposure (Pellet *et al.*, 1995; Ma *et al.*, 1997a; Li *et al.*, 2000; Ma *et al.*, 2000; Yang *et al.*, 2000).

In plants exhibiting the first pattern of exudation, the capacity for synthesis of organic anions appears to remain relatively unchanged. In a resistant variety of wheat that exudes malate upon Al exposure, no differences were measured in the activities of key enzymes of the TCA cycle (phosphoenolpyruvate-carboxylase (PEPC), NADP⁺-dependent isocitrate dehydrogenase (NADP⁺-ICDH), NAD-malate dehydrogenase (NAD-MDH), and citrate synthase (CS) (Ryan *et al.*, 1995b; Li *et al.*, 2000). On the other hand, changes in the activities of TCA cycle enzymes have been observed in plants exhibiting the second pattern of organic anion exudation. A 30% increase in CS activity was measured in Al-resistant rye plants after a 6 h exposure to Al, and citrate efflux followed 4 h later (Li *et al.*, 2000). Activities of PEPC, NADP⁺-ICDH, and NAD-MDH were not affected. The hypothesis linking Al-induced biosynthetic processes to resistance mechanisms is also supported by additional experimental evidence. Through radiolabelling studies, Basu *et al.* (1994) observed *de novo* synthesis of malate in wheat in response to Al. In maize plants, a transient accumulation of Al and/or callose in root tips has also been temporally correlated with the lag time for expression of Al resistance. This suggests that resistance mechanisms are induced upon exposure to Al (Jorge and Arruda, 1997; Vázquez *et al.*, 1999; Gunsé *et al.*, 2000).

1.2.8. Genetic manipulation of organic anion efflux

The development and study of transgenic plants resistant to Al has provided additional evidence supporting the second pattern of organic anion exudation. Several groups have cloned genes encoding enzymes involved in organic anion metabolism and have used these to manipulate biosynthetic pathways in plants. de la Fuente *et al.* (1997)

overexpressed a bacterial CS gene in the cytoplasm of tobacco and papaya plants, and observed an increase in internal citrate pools and citrate efflux, and a subsequent decline in root growth inhibition by Al. However, Delhaize *et al.* (2001) attempted to repeat these experiments in tobacco and did not observe enhanced citrate release, internal citrate pools, or Al-resistance, even though they observed a 100 fold increase in CS protein in the plants. They suggested that this system was not easily reproducible and may be sensitive to environmental conditions. Nonetheless, Takita *et al.* (1999a, 1999b) and Koyama *et al.* (1999) overexpressed an *Arabidopsis thaliana* mitochondrial CS in carrot cells and observed a moderate increase in citrate efflux and resistance to Al. Transgenic *Arabidopsis thaliana* with enhanced CS activity, also showed increased internal citrate concentrations and citrate efflux (Koyama *et al.*, 2000). Likewise, a 1.6 fold increase in MDH activity of transformed alfalfa plants increased both malate pools in plant tissues and organic anion exudation, and enhanced resistance to Al (Tesfaye *et al.*, 2001). Overexpression of PEPC in these same plants did not result in increased Al resistance. Recently Anoop *et al.* (2003) reported that the overexpression of mitochondrial CS in canola resulted in up to a 2 fold increase in CS transcript expression, enhanced levels of CS enzyme activity, cellular shoot citrate, and citrate exudation. The transgenic lines of canola also showed greater root elongation in the presence of Al when compared with the wildtype.

As a group, these studies support the idea that organic anions play an important role in Al resistance. They also suggest that exposure to Al may trigger a pathway leading to the induction of metabolic processes and enhanced resistance in certain plants. However,

studies have not yet shown a clear correlation in time between the induction of resistance mechanisms and the expression of an Al-resistant phenotype. If resistance is not constitutively expressed, delays in the phenotypic expression of resistance should coincide with delays in gene expression and subsequent organic anion exudation. In the future, attention needs to be focused on further characterizing plant responses over the period of induction to clarify if a relationship truly does exist between potential resistance mechanisms and the recovery from Al injury.

1.3. Summary

This research was undertaken to investigate the induction of Al resistance in the snapbean cultivar Dade. The goal of this research was to characterize root responses to Al and to examine Al-stimulated citrate efflux over the period of induction. To achieve this goal, specific objectives were addressed:

- (1) Confirm the differential Al resistance between Al-sensitive (Romano) and Al-resistant (Dade) cultivars of snapbean.
- (2) Confirm the induction of Al resistance in the snapbean cultivar Dade.
- (3) Develop an experimental system to measure Al-induced exudation of citrate in snapbean.
- (4) Characterize the induction of Al resistance and the subsequent recovery from Al injury in Dade by examining citrate exudation and root response (callose production, root elongation, cellular morphology, hematoxylin staining) to Al over time.

This research was designed to test the hypothesis that Al-resistance is induced in cv. Dade. Stronger support for the organic anion hypothesis of Al resistance would be provided if citrate exudation was correlated with the recovery from Al injury in Dade. Continued study of the responses of resistant plants to Al is required to elucidate mechanisms controlling Al resistance. This research will contribute to the further understanding of the physiology and biochemistry of Al toxicity and resistance in plants and may aid in the development of Al-resistant crops that achieve greater productivity on acid soils throughout the world.

2. MATERIALS AND METHODS

2.1. High Volume Studies

2.1.1. Plant material and culture conditions

Seeds of two snapbean (*Phaseolus vulgaris* L.) cultivars, Dade (Al-resistant) and Romano (Al-sensitive), were nicked with a razor blade and rolled in damp paper towels. Paper towels were placed upright in 2 L of 1 mM CaCl₂ (pH 4.3) containing 0.005 g L⁻¹ Vitavax to limit fungal growth. Seed coats of germinated seeds were removed after 4 d in the dark. Seedlings were mounted onto perforated (1 cm diameter holes) black Plexiglas plates suspended in aquaria over 10 L of aerated nutrient solution (pH 4.3) consisting of: (mM) 3.30 NO₃⁻-N, 0.30 NH₄⁺-N, 0.10 P (PO₄³⁻), 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S, (μM) 34 Cl, 20.2 Na, 10 Fe, 6 B, 1 Mn, 0.15 Cu, 0.5 Zn, 0.10 Mo, 10 EDTA. Solution pH was adjusted to 4.3 with 1 M HCl. Germination and seedling growth were carried out in a controlled environment chamber maintained at an average photosynthetic photon flux density of 350 ± 50 μmol m⁻² s⁻¹, 16 h/22 °C/60% humidity days and 8 h/19 °C/80% humidity nights.

2.1.2. Long-term dose response

Seedlings were grown in covered aquaria for 4 (Dade) or 5 d (Romano) to generate plants of equal size. Stems were wrapped in foam (1 per foam), mounted onto Plexiglas plates, and seedlings were suspended over pots containing 10 L of aerated nutrient solution. A randomized complete block design was used with 3 blocks (replicates), 2 cultivars, and 10 levels of AlCl₃ (0, 10, 20, 40, 70, 100, 200, 300, 400, 600 μM) at pH 4.3 (60

experimental units). Initial pH was adjusted to 4.3 with 1 M HCl. Acid was added to nutrient solutions before addition of the AlCl_3 stock solution (100 mM in 1 mM HCl) to avoid precipitation of Al. Free Al^{3+} in Al solutions was predicted (GEOCHEM-PC, version 2.0) to be 0, 1.7, 3.5, 7.3, 14.2, 15.7, 16.0, 15.9, 15.8, and 16.0 μM . The experiment was performed in a controlled environment chamber maintained at an average photosynthetic photon flux density of $335 \pm 51 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16 h/26 °C/60% humidity days and 8 h/21 °C/80% humidity nights. Over the 16 d experimental period, pH and electrical conductivity (EC) were monitored, and pH was adjusted to 4.3 every other day using 0.1 M HCl. On days 8 and 12, 5.0 mL of 0.6 M NH_4NO_3 was added to each treatment solution to assist in pH control. At the end of the experimental period, plants were harvested, separated into roots and shoots, dried in an oven at 60 °C and weighed.

2.1.3. Time course of primary root response to aluminum

Seedlings of both Dade and Romano were grown in covered aquaria for 4 d. Primary root length of seedlings was measured prior to transfer to treatment solutions (0 h). Stems were wrapped in foam (1-2 per foam), mounted onto Plexiglas plates, and seedlings were suspended over pots containing 10 L of aerated nutrient solution (pH 4.3). A repeated measures design was used with 15 blocks (replicates), 2 cultivars, and 2 levels of AlCl_3 (0, 50 μM) (60 experimental units). In each experiment there were 5 or 8 time treatments (3, 6, 9, 12, 24 h) or (1, 2, 3, 4, 5, 6, 7, 9 d) per experimental unit. Initial pH was adjusted to 4.3 with 1M HCl. Acid was added to nutrient solutions before addition of the AlCl_3 stock solution (100 mM in 1 mM HCl) to avoid precipitation of Al. Free Al^{3+} in Al solutions was predicted (GEOCHEM-PC, version 2.0) to be 9.6 μM . Both pH and

electrical conductivity were monitored daily throughout the experiment. The pH of treatment solutions was adjusted daily to 4.3 using 0.1 M HCl. Plants were harvested at each treatment time and primary root length of each seedling was measured. Root elongation was expressed as the change in length from 0 h to a particular time treatment ($\text{length}_{\text{time } x} - \text{length}_{\text{time } 0}$). The experiment was performed in a controlled environment chamber maintained at an average photosynthetic photon flux density of $373 \pm 80 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16 h/26 °C/60% humidity days and 8 h/21 °C/80% humidity nights.

2.2. Low Volume Studies

2.2.1. Plant material and culture conditions

Seeds of two snapbean (*Phaseolus vulgaris* L.) cultivars, Dade (Al-resistant) and Romano (Al-sensitive), were surface sterilized by soaking in 70% ethanol for 30 s, then in a 2% sodium hypochlorite solution with Tween 20 (3 drops per 150 mL) for 10 min, followed by rinsing in sterile water. Seeds were germinated in the dark for 4 d in Petri dishes containing sterile paper towels dampened with 1.0 mM CaCl_2 (pH 4.3). Seed coats of germinated seeds with no evidence of fungal or bacterial contamination were removed. These seeds were then transferred to canvas rafts suspended over 45 mL of aerated pretreatment solution (0.4 mM CaCl_2 , pH 4.3) in the apparatus described below for 24 h.

Seedlings were grown under aseptic conditions in an apparatus adapted from Miyasaka *et al.* (1991). Two magenta vessels were fitted together with a connector to create a closed, aseptic system. A 50 mL polypropylene centrifuge tube containing treatment solution was placed in the bottom magenta vessel, supported by foam. Small rafts were cut from

plastic canvas and inserted into the opening of the centrifuge tube to support seedlings (one per tube). A 0.45 μm syringe air filter was fitted into the top magenta vessel, and sealed with silicone. A piece of Teflon tubing (OD 4 mm, ID 2.5 mm) reaching the bottom of the centrifuge tube was attached to the filter to allow for sterile aeration of the treatment solution. Supplies and solutions were sterilized using an autoclave or filter sterilization, and all transfers were performed in a sterile hood. In addition, supplies were washed in a solution of 2% (v/v) HNO_3 and rinsed in milli-Q water prior to sterilization. Germination and seedling growth were carried out in a controlled environment chamber maintained at $133 \pm 17 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density, 16 h/21 °C/60% humidity days and 8 h/ 20 °C/80% humidity nights.

2.2.2. Time course experiments

Following the 24 h pretreatment in 0.4 mM CaCl_2 solution (pH 4.3), rafts were transferred to 50 mL tubes in the aseptic culture system containing 45 mL of 0.4 mM CaCl_2 (pH 4.3) with and without Al. A randomized design was used with 2 cultivars (Dade, Romano), 2 Al levels (0, 25 μM AlCl_3), and 3-4 time treatments (3, 6, 12 h) or (24, 48, 72, 96 h), with 5 replicates for each treatment (60 or 80 experimental units). To prevent precipitation of Al caused by pH changes during autoclaving, AlCl_3 solutions were filter sterilized through 0.45 μm filters. Free Al^{3+} in Al solutions was predicted (GEOCHEM-PC, version 2.0) to be 0 or 14.5 μM . At each harvest, 200 μL of treatment solution was plated onto Luria-Bertani broth (LB) containing 15 mg mL^{-1} agar to monitor microbial contamination. Primary root length was measured prior to transfer (0 h) to treatment solutions and at each harvest. Root elongation was expressed as the change in

length from 0 h to a particular time treatment ($\text{length}_{\text{time } x} - \text{length}_{\text{time } 0}$). Final pH was measured at each harvest and 5 mL of treatment solution was withdrawn from each tube and stored at $-80\text{ }^{\circ}\text{C}$ until citrate was analyzed. An additional 10 mL of treatment solution was withdrawn for analysis of Al. The apical 1 cm of the primary root was excised, weighed, and fixed in 1 mL of 95% ethanol ($4\text{ }^{\circ}\text{C}$) for at least 16 h for callose determination. The remaining roots were harvested, dried at $60\text{ }^{\circ}\text{C}$ and weighed.

2.2.3. Enzymatic measurement of citrate

Samples of treatment solutions were thawed and acidified to $\text{pH} < 2.0$ with 6 N HCl to dissociate citrate from Al-citrate compounds. Standards were made in 0.4 mM CaCl_2 ($\text{pH } 2.0$) by serial dilution of a 100 μM citric acid stock. Each sample and standard was then passed through a cation exchange column filled with AG-50W-X8 cation exchange resin (H^+ -form, BioRad), dried under a stream of air at $60\text{ }^{\circ}\text{C}$, and resuspended in 300 μL of milli-Q water. Citrate concentration in the eluate was measured using modifications of the method of Dagley (1974). Each sample or standard (100 μL) was incubated with 1.0 mL of a mixture of buffer (0.1 M Tris, $\text{pH } 8.2$), 0.1 mM ZnCl_2 , 0.1 mM $\beta\text{-NADH}$, and malate dehydrogenase (3 U mL^{-1}) for 13 min, and an initial absorbance reading at 340 nm was obtained. Ten μL of citrate lyase (0.126 U mL^{-1} dissolved in 0.1 M Tris) was then added, and the solution was mixed and incubated for 10 min. A final absorbance reading was taken at 340 nm. The decline in absorbance (A_{340}) due to the oxidation of NADH was directly proportional to the amount of citric acid in the sample. Results for citrate exudation were expressed as nmoles citrate exuded per gram dry root mass.

2.2.4. Spectrofluorometric determination of callose

Callose was determined by the method of Kohle *et al.* (1985) as modified by Zhang *et al.* (1994). The 95% ethanol used to fix the 1 cm root tip collected for callose determination was decanted and replaced with 0.2 mL of 1N NaOH. After a brief incubation period (5-10 min), roots were ground (15-20 s) using a Teflon pestle mounted onto an electric drill. After each grinding, the pestle was rinsed into the sample tube with 0.3 mL of 1 N NaOH. Standards were made by serial dilution of a β -1,3-glucan, Pachyman (*Poria cocos*; Calbiochem), stock solution (2 mg in 100 mL 1 N NaOH). Samples and standards were incubated for 15 min at 80 °C to solubilize the callose, and then centrifuged for 4 min at 15,000 x g. An aliquot (0.4 mL) of the supernatant or standard was mixed with 0.8 mL aniline blue (0.1% w/v), 0.42 mL 1N HCl, and 1.18 mL 1M glycine-NaOH buffer (pH 9.5) and incubated for 20 min at 50 °C, followed by a 30 min incubation at 25 °C. Callose content was determined spectrofluorometrically at an excitation wavelength of 398 nm and an emission wavelength of 495 nm. Callose content was expressed as μ g pachyman equivalents per gram fresh root mass.

2.2.5. Determination of total aluminum in solutions

Total Al content of experimental solutions was determined after each harvest by the methods of Zhang and Taylor (1989) using a graphite-furnace atomic absorption spectrophotometer (GFAAS). A sample was mixed with 20 μ L of a matrix modifier ($\text{Mg}(\text{NO}_3)_2$), dried at 150 °C for 60 s, pretreated at 1700 °C for 45 s, and atomized at 2500 °C for 5 s on a L'vov platform in a pyrolytically coated graphite tube. Aluminum

concentrations were calculated by integration of peak area. All supplies were washed with HNO_3 (2% v/v) and rinsed with milli-Q water.

2.2.6. Determination of monomeric Al in solutions

Inorganic monomeric Al content of experimental solutions was determined after each sampling time using the pyrocatechol violet (PCV) colorimetric method (Kerven *et al.*, 1989a; Menzies *et al.*, 1992). A 60 s reaction time was used due to the presence of organic ligands in solution (Kerven *et al.*, 1989b). Standards were prepared by dilution of a 1,000 mg L⁻¹ Al reference solution in 1.0 mM HCl. Three mL of the solution or standard was mixed with 0.5 mL of an iron interference reagent (5 mM 1,10-phenanthroline and 28.4 mM L-ascorbic acid). Following an incubation of 60 s, 0.2 mL of PCV reagent (1.9 M) and 1.0 mL of imidazole buffer (1 M, pH 5.6) were added to the mixture. Tubes were shaken lightly and absorbance at 578 nm was recorded following a 60 s reaction time. Analytical procedures were carried out using supplies washed in dilute HNO_3 (2% v/v) and rinsed with milli-Q water.

2.3. Morphological Studies

2.3.1. Plant material and culture conditions

Seeds were germinated and grown in a high volume system as described in sections 2.1.1 and 2.1.3. A randomized complete block design was used with 3 blocks (replicates), 2 cultivars, 2 levels of AlCl_3 (0 and 50 μM , pH 4.3), and 5 time treatments (1, 2, 3, 4, 5 d) (60 experimental units). Ten mL of treatment solution was withdrawn from each pot for monomeric Al analysis as described in section 2.2.6. The apical 1 cm of root tips in each

cultivar*Al* time combination, and 1.5 cm segments along the entire primary root in each cultivar*Al combination at 4 d were excised. Three replicates of each were fixed in Randolph's Modified Navashin Fluid (Craf) (Johansen, 1940) under vacuum at 25 °C for 24 h and used in microscopic studies. In addition, whole roots of three replicates of each cultivar*Al* time combination were excised from stems for hematoxylin staining.

2.3.2. Microscopic observation

Excised root material was removed from the fixative, transferred to capsules and washed under running water for 2 h. The tissue was dehydrated in a graded ethanol (ETOH) series (10% – 1 h, 20% – 1 h, 30% – 1 h, 50% – 1 h, 70% – 12 h) followed by a graded ETOH/tertiary butyl alcohol (TBA) series (70% – 12 h, 85% – 1 h, 95% – 1 h, 100% – 1 h, pure TBA – 3 h). Root segments were transferred to a 1:1 mixture of TBA and paraffin oil for 12 h (Johansen, 1940). The tissue was then infiltrated with paraffin wax and embedded in paraffin blocks. Blocks were allowed to harden for 24 h, trimmed, mounted in a microtome, and longitudinal sections (10 µm) were cut. Ribbons of sections were mounted onto glass slides by coating a thin layer of Haupt's adhesive (Johansen, 1940) onto a clean slide, flooding the surface with 4% formalin, and placing sections onto the formalin. Slides were placed on a slide warmer until wax ribbons were adequately stretched, then excess formalin was drained. After drying slides for at least 24 h at 37 °C, wax was removed with toluene. Slides were mounted with cover slips using DePeX (BDH) mounting medium and dried at 37 °C for 24 h. Unstained tissue was observed at either a 16 X (root segments along entire root) or 40 X (root tips) magnification using differential interference contrast (DIC) microscopy to study cortical cell morphology and

alignment. Observations were focused on the outer 4-6 cortex cell files of root segments. In the 1 cm root tip segments images were taken in the region 0.15-0.25 cm from the root cap. In studies of the entire root images were taken at the halfway point (0.75 cm) of each 1.5 cm root segment.

2.3.3. Hematoxylin staining of primary roots

Hematoxylin (Natural Black 1, Sigma) (2 g L^{-1}) was dissolved in sodium iodate (NaIO_3) (0.2 g L^{-1}) for 24 h and roots were stained following the procedure of Polle *et al.* (1978). Excised roots were rinsed and washed in dH_2O for 30 min. Roots were then stained with the hematoxylin solution for 15 min, rinsed, and once again washed in dH_2O for 30 min. Washing solutions were changed twice over each 30 min period. Images were taken of primary roots and root tips.

2.4. Statistical Analysis

Analysis of variance (ANOVA) and least squares means (LSM) were calculated using Statistical Analysis Systems Institute software (SAS, release 8.2). A probability value (P) of 0.05 or less was considered statistically significant.

3. RESULTS

3.1. Analysis of Plant Response to Aluminum in a High Volume System

3.1.1. Long-term dose response

A long-term (16 d) dose response experiment was conducted in a high volume (10 L) growth system to measure the resistance of snapbean cultivars Dade and Romano to varying levels of Al (0, 10, 20, 40, 70, 100, 200, 300, 400, and 600 μM AlCl_3 , pH 4.3). Root and shoot dry weight were used as measures of Al response (Figure 1). Dade and Romano shoots showed similar dry mass under control conditions, and in the 10 μM , and 20 μM Al treatments. Dade shoots exhibited significantly higher dry mass in all other Al treatments (Figure 1a). Maximal differences between the two cultivars were observed at 200 μM Al, where dry mass of Dade shoots was 3 fold more than Romano shoots (Figure 1a, 2). Shoot dry mass of cv. Romano was reduced by Al treatments greater than 10 μM Al and continued to decrease progressively in concentrations of up to 300 μM Al. No further reduction was observed after 16 d of exposure to higher levels of Al. Contrarily, shoot dry weight of Dade was adversely affected by Al concentrations exceeding 100 μM and progressively declined up to 400 μM Al. No further reduction in shoot dry mass was observed in treatments of 400 μM Al and more (Figure 1a).

Dade and Romano showed similar root dry mass under control conditions and when exposed to 10 μM Al. Dry mass of Dade roots was significantly greater than dry mass of Romano roots in the 20, 40, 70, 100, and 200 μM Al treatments (Figure 1b). Maximal differences occurred between the two cultivars when exposed to Al concentrations

between 40-100 μM , where Dade exhibited up to 2 fold greater root dry mass than Romano (Figure 1b, 2). Romano roots began showing Al toxicity symptoms at concentrations exceeding 10 μM Al, and dry mass continued to decline in plants exposed to treatment solutions containing up to 300 μM Al. No further reductions in root dry mass were observed at concentrations of 300 μM Al and above. Romano roots were brown, brittle, and stunted, with limited lateral root growth after a 16 d exposure to 100 μM Al (Figure 2b). In contrast, root dry mass in Dade declined upon exposure to Al concentrations greater than 40 μM and declined progressively in treatments of up to 400 μM Al. No further reduction was observed after 16 d of exposure to higher levels of Al (Figure 1b). Aluminum toxicity symptoms in Dade roots only became visible upon exposure to 200 μM Al (Figure 2a).

3.1.2. Time course of primary root response to aluminum

The primary root response of cv. Dade and cv. Romano to Al was measured over time in a high volume experimental system. Due to size limitations of the experimental system, separate experiments were conducted to measure response at 3, 6, 9, 12, and 24 h (Figure 3a) and 1, 2, 3, 4, and 5 d (Figure 3b). A concentration of 50 μM AlCl_3 was used in Al solutions, and primary root elongation ($\text{length}_{\text{time } x} - \text{length}_{\text{time } 0}$) was used as a measure of Al injury. Trend analysis of the significant Al*cultivar interaction using LSM showed that the overall linear trend was significant for primary root elongation over both the 3-24 h and 1-5 d time periods. The linear trend over the 3-24 h period of both Dade and Romano cultivars treated with 50 μM Al was significantly different from that of corresponding plants grown under control conditions (Figure 3a). In cv. Dade, primary

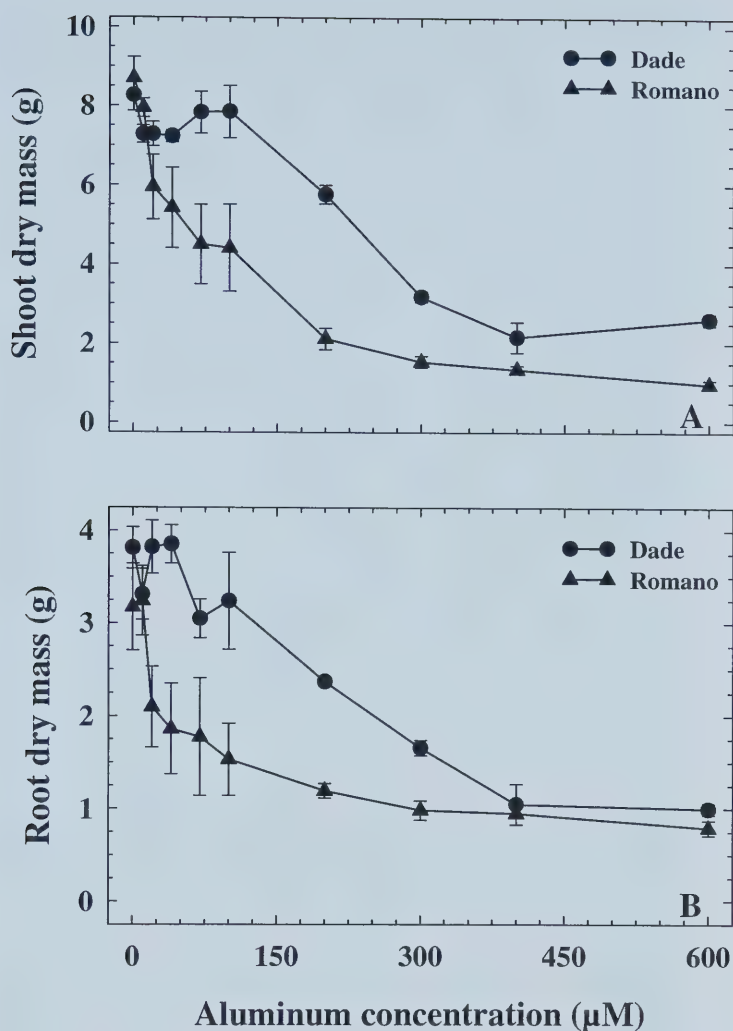


Figure 1. Long-term (16 d) dose response of *Phaseolus vulgaris* L. to aluminum. Cultivars Dade and Romano were exposed to 0, 10, 20, 40, 70, 100, 200, 300, 400, and 600 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3). Shoot (A) and root (B) dry masses were used to measure the toxic response to Al. A randomized complete block design was used with 3 blocks, 2 cultivars, and 10 levels of AlCl_3 . Values represent the means $\pm$ SE of three replicates.



Figure 2. Differential resistance of Dade and Romano to aluminum. Images of cultivars (A) Dade and (B) Romano roots exposed to 0, 100, 200, 300, and 600 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3).

roots treated with 50 μ M Al only exhibited 68% of control elongation after 1 d (Figure 3a). However, elongation of Al-treated primary roots of Dade then recovered to levels similar to controls (Figure 3b). In addition, primary roots of Dade elongated up to 3 times more than those of Romano over the rest of the experimental period (Figure 3b). Only the linear trend for primary root elongation of Romano roots exposed to 50 μ M Al was significantly different from all other treatments after 1-5 d of treatment (Figure 3b).

3.1.3. Determination of monomeric aluminum and pH in treatment solutions

Soluble, monomeric Al (μ M) was measured in treatment solutions using the PCV assay to verify that substantial Al depletion did not occur over the experimental period.

Solution samples were collected and tested before daily pH adjustment to get an accurate representation of Al remaining in solutions. Aluminum treatment solutions of both cultivars always contained significantly more monomeric Al than control solutions.

Soluble Al in 50 μ M Al solutions decreased over the first day of treatment by 34% and 39% in Romano and Dade, respectively, then levels remained constant over the 5 d experimental period (Table 1). Solution pH values were also measured before daily readjustment (Table 2). No significant pH differences were apparent between cultivars in Al solutions over time. On the other hand, Romano plants under control conditions altered pH significantly more than Dade plants at 3, 4, and 5 d. In cv. Dade, the pH of control and Al solutions ranged from 4.3-4.5. The pH of Romano control solutions increased slightly over time, with values reaching a maximum of pH 4.7 at 4 d. In contrast, the pH of Al treatment solutions was never more than 4.4 (Table 2).

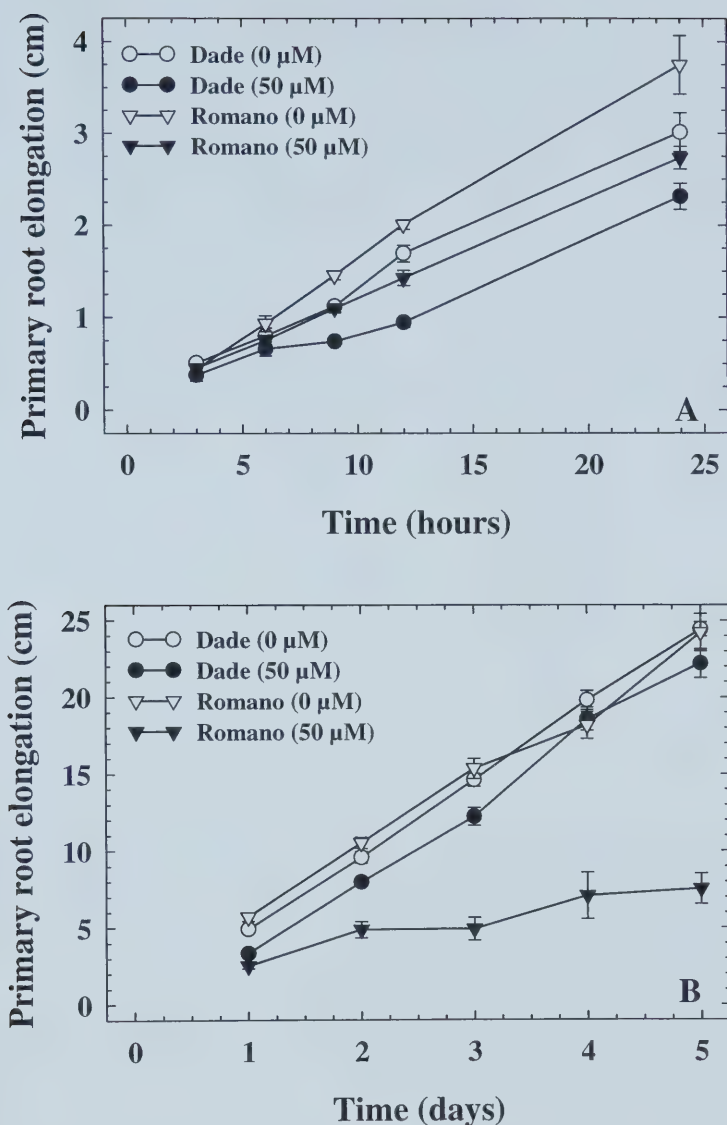


Figure 3. The induction of aluminum resistance in Dade (*Phaseolus vulgaris* L.) over time. Plants were exposed to 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) over (A) 3, 6, 9, 12, and 24 h and (B) 1, 2, 3, 4, and 5 d. Primary root elongation ($\text{length}_{\text{time } x} - \text{length}_{\text{time } 0}$) was used as a measure of Al injury. A repeated measures experimental design was used with 15 blocks, 2 cultivars, and 2 levels of AlCl_3 . Values represent the means $\pm$ SE of fifteen replicates.

Table 1. Determination of monomeric aluminum in treatment solutions. Soluble Al (μM) present in 10 L of full nutrient solution at 0 d (before planting), and after 1, 2, 3, 4, and 5 days of treatment, as measured by PCV. Solution samples were taken before daily pH adjustment. A random complete block experimental design was used with 3 blocks, 2 cultivars, 2 levels of AlCl_3 , and 5 time treatments. Values represent the means $\pm$ SE of three replicates.

Time (days)	Dade (0 μM)	Dade (50 μM)	Romano (0 μM)	Romano (50 μM)
0	2.2 $\pm$ 0.03	11.4 $\pm$ 0.24	2.2 $\pm$ 0.02	11.5 $\pm$ 0.19
1	1.8 $\pm$ 0.01	7.0 $\pm$ 0.37	1.8 $\pm$ 0.01	7.7 $\pm$ 0.17
2	1.8 $\pm$ 0.01	6.7 $\pm$ 0.14	1.9 $\pm$ 0.02	9.2 $\pm$ 0.68
3	1.9 $\pm$ 0.01	6.8 $\pm$ 0.53	1.9 $\pm$ 0.00	8.8 $\pm$ 0.49
4	1.9 $\pm$ 0.02	7.3 $\pm$ 0.14	1.9 $\pm$ 0.01	8.4 $\pm$ 0.09
5	1.9 $\pm$ 0.04	8.4 $\pm$ 1.19	1.9 $\pm$ 0.02	8.3 $\pm$ 0.44

Table 2. Determination of pH changes in treatment solutions over time. Measurement of pH in treatment solutions (0 and 50 μM AlCl_3 in 10 L of full nutrient solution, pH 4.3) over time (0, 1, 2, 3, 4, 5 d). Values were recorded before daily readjustment to pH 4.3. A random complete block experimental design was used with 3 blocks, 2 cultivars, 2 levels of AlCl_3 , and 5 time treatments. Values represent the means $\pm$ SE of three replicates.

Time (days)	Dade (0 μM)	Dade (50 μM)	Romano (0 μM)	Romano (50 μM)
0	4.3 $\pm$ 0.00	4.3 $\pm$ 0.00	4.3 $\pm$ 0.00	4.3 $\pm$ 0.00
1	4.4 $\pm$ 0.00	4.4 $\pm$ 0.01	4.4 $\pm$ 0.01	4.4 $\pm$ 0.01
2	4.4 $\pm$ 0.01	4.4 $\pm$ 0.00	4.4 $\pm$ 0.01	4.3 $\pm$ 0.00
3	4.4 $\pm$ 0.01	4.4 $\pm$ 0.01	4.5 $\pm$ 0.04	4.3 $\pm$ 0.01
4	4.5 $\pm$ 0.01	4.3 $\pm$ 0.01	4.7 $\pm$ 0.03	4.4 $\pm$ 0.01
5	4.4 $\pm$ 0.01	4.3 $\pm$ 0.03	4.6 $\pm$ 0.02	4.3 $\pm$ 0.04

3.2. Analysis of Plant Response to Aluminum in a Low Volume System

A low volume (45 mL), aseptic culture system with simple salt solutions was developed to measure citrate exudation by *Phaseolus vulgaris* L. in response to Al. A concentration of 25 μM AlCl_3 (pH 4.3) was used in this system due to the low ionic strength of the growth solution (0.4 mM CaCl_2). As a result of material and size constraints of this system, separate experiments were conducted to measure plant responses to Al over 3-12 h, and 1-4 d.

3.2.1. Time course of aluminum-induced citrate exudation

Dade and Romano roots grown in both control and Al solutions exuded similar amounts of citrate over the 3-12 h experimental period (Figure 4a) in the low volume system. On the other hand, significant differences in citrate exudation were measured between the 0 and 25 μM Al treatments over the 1-4 d experimental period. Romano and Dade roots treated with 25 μM Al exuded up to 20 and 27 fold more citrate, respectively, than those in control solutions after 4 d of exposure (Figure 4b). Furthermore, the snapbean cv. Dade exuded significantly more citrate than cv. Romano at all times when exposed to 25 μM Al over 1-4 d. Roots of cv. Dade treated with Al exuded up to 3 times more citrate than Romano roots (Figure 4b).

3.2.2. Time course of plant response to aluminum

Dade and Romano plants exhibited similar primary root elongation over time in all treatments in the low volume system (Figure 5a, b). However, plants grown in control solutions showed greater root elongation than those grown in 25 μM Al from 6-12 h and

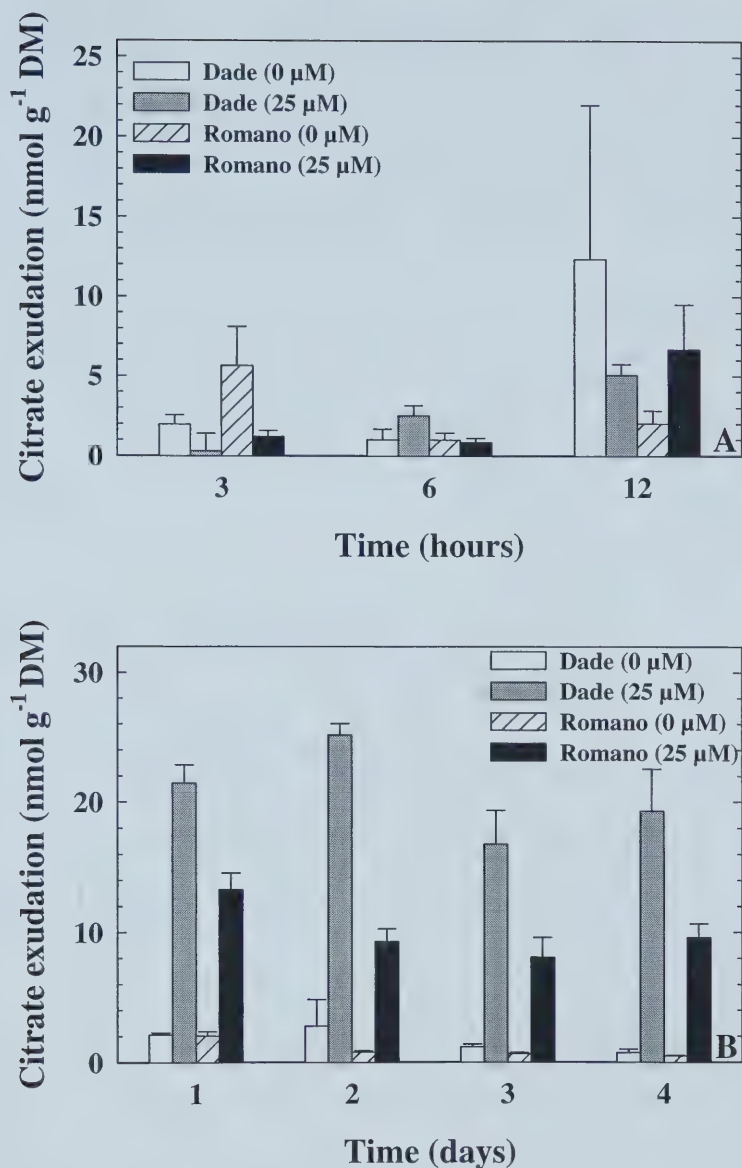


Figure 4. Time course of aluminum-induced citrate exudation. Citrate exudation of roots of Dade and Romano in response to 0 or 25 μM AlCl₃ in 45 mL of 0.4 mM CaCl₂ (pH 4.3) over (A) 3, 6, and 12 h and (B) 1, 2, 3, and 4 d. A randomized experimental design was used with 2 cultivars, and 2 levels of AlCl₃, and 3-4 timed harvests. Values represent the means ± SE of five replicates.

1-4 d. Dade roots exhibited 39% of control elongation after 12 h of Al exposure, but recovered to 74% of control elongation at the end of the 4 d treatment. Romano roots showed a similar pattern of inhibition and recovery of elongation in response to Al over time. New tip growth of both cultivars did not show typical symptoms of Al injury as primary root growth recovered over time (Figure 6). Although shoot growth was not a measured parameter in this study, visual observations suggest that shoot growth and maturation of Romano may still be sensitive to Al after 3 d of treatment in the low volume system (Figure 6).

3.2.3. Time course of aluminum–induced callose deposition

There were no significant differences between cultivars in callose production over both the 3-12 h and 1-4 d experimental periods in the low volume system. Roots of both cultivars treated with 25 μ M Al accumulated significantly higher levels of callose than controls over the 3-12 h time period and after 1 d of treatment (Figure 7a, b). For example, roots of both Dade and Romano exposed to 25 μ M Al produced up to 10 times more callose than those grown in control solutions for 3-12 h. Callose deposition subsequently declined following 1 d of treatment. Roots grown in control and Al solutions accumulated similar levels of callose over the remainder of the experimental period (2-4 d).

3.2.4. Determination of total and monomeric aluminum remaining in solutions

Levels of total and monomeric Al were measured in treatment solutions to determine if depletion of toxic Al species was occurring in the low volume experimental system.

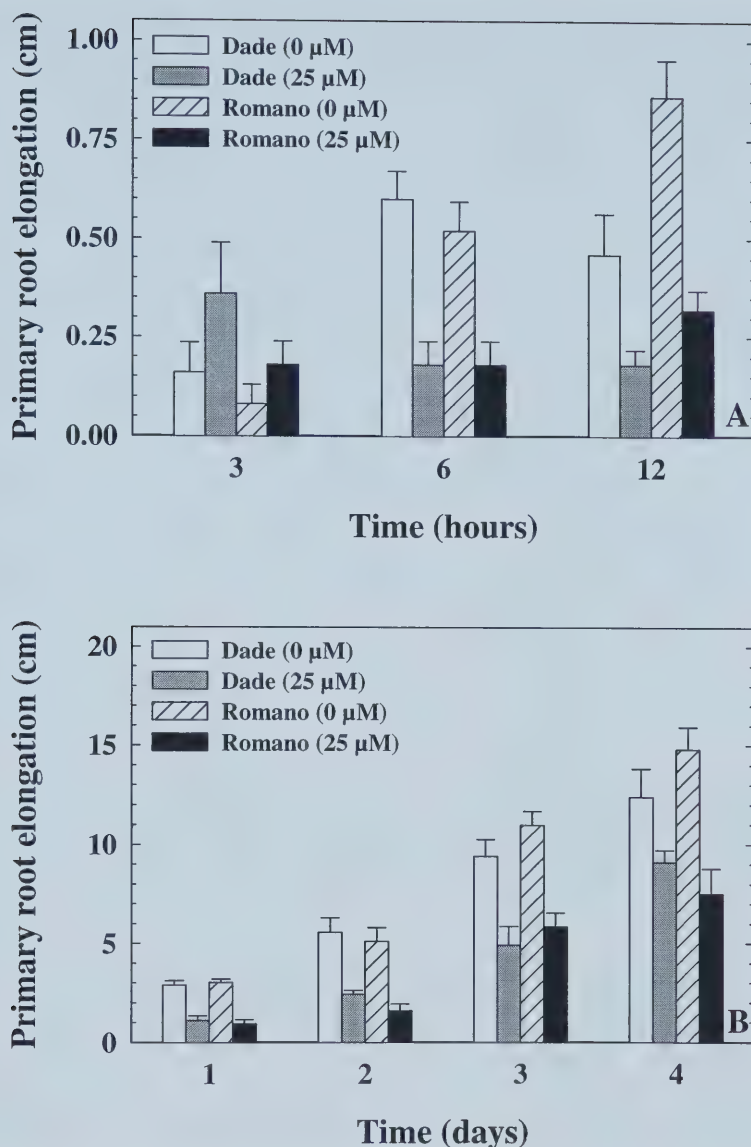


Figure 5. Time course of primary root response to aluminum in the low volume solutions. The response of cultivars Dade and Romano (*Phaseolus vulgaris*) to 0 or 25 μM AlCl_3 in 45 mL of 0.4 mM CaCl_2 (pH 4.3) over (A) 3, 6, and 12 h and (B) 1, 2, 3, and 4 d. Primary root elongation ($\text{length}_{\text{time } x} - \text{length}_{\text{time } 0}$) was used as a measure of Al injury. A randomized experimental design was used with 2 cultivars, 2 levels of AlCl_3 , and 3-4 timed harvests. Values represent the means $\pm$ SE of five replicates.



Figure 6. Recovery of primary root elongation in *Phaseolus vulgaris* L. grown in a low volume experimental system. Dade and Romano plants were exposed to 0 or 25 μM AlCl_3 in 45 mL of 0.4 mM CaCl_2 (pH 4.3) for three days. Primary roots of Al treated plants resumed growth and tips showed no symptoms of Al injury.

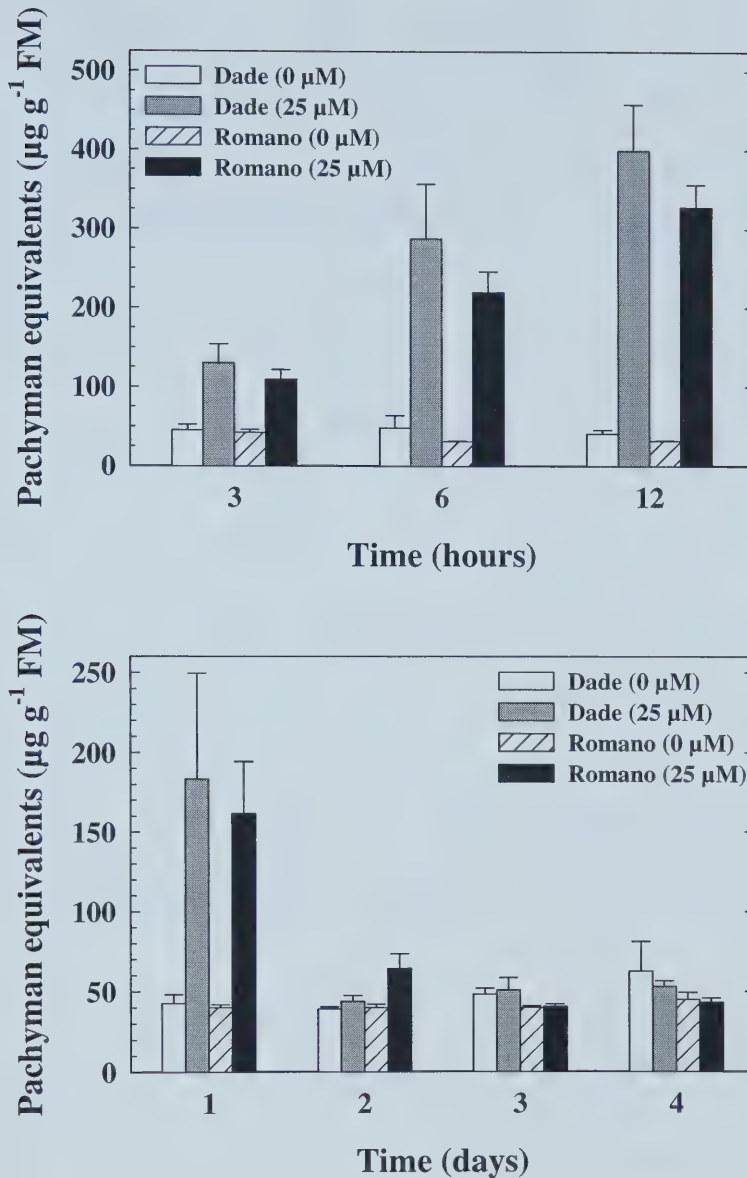


Figure 7. Time course of aluminum-induced callose deposition. Callose content, expressed as μg Pachyman equivalents per gram of fresh root mass, in root tips of Dade and Romano in response to 0 or 25 μM AlCl_3 in 45 mL of 0.4 mM CaCl_2 (pH 4.3) over (A) 3, 6, and 12 h and (B) 1, 2, 3, and 4 d. A randomized experimental design was used with 2 cultivars, and 2 levels of AlCl_3 , and 3-4 timed harvests. Values represent the means $\pm$ SE of five replicates.

Aluminum solutions of both cultivars (Table 3) had significantly more total Al than control solutions (data not shown) at each time point. Total Al in 25 μ M solutions of both cultivars declined substantially, but there were no significant differences between cultivars in the total Al content of treatment solutions over the 3-12 h period (Table 3a). On the other hand, Al solutions of Dade contained significantly more total Al than Romano solutions during the 1-4 d experimental period. In addition, total Al in 25 μ M solutions of Dade increased over 1-3 d (Table 3b).

Although Al solutions of Dade and Romano contained significantly more monomeric Al (Table 3) than control solutions (data not shown) from 3-12 h, Al levels in 25 μ M treatment solutions declined to levels similar to controls over the 1-4 d period. Monomeric Al declined considerably in the first 6 h of treatment in 25 μ M Al solutions of both Dade and Romano. There were no significant differences between cultivars in the monomeric Al content of 25 μ M solutions over the 3-12 h or 1-4 d experimental periods (Table 3a, b).

3.2.5. Comparison of citrate exuded to total aluminum in solutions

The ratio of total citrate to total Al content (citrate:total Al) was examined to determine the maximum amount of Al that was theoretically bound to citrate in treatment solutions. Citrate:total Al in 25 μ M solutions increased over time in both cultivars from 3-12 h (Table 3a). This pattern continued in Dade from 1-4 d, while citrate:total Al in Romano varied over this time period (Table 3b). In addition, 25 μ M Al solutions of Dade showed lower citrate:total Al than Romano at each time point (Table 3a, b).

Table 3. Absolute levels of total aluminum, monomeric aluminum, citrate, and the ratio of citrate to total aluminum in treatment solutions. Total Al (as measured by GFAAS), monomeric Al (as measured by PCV), and total citrate in 45 mL of treatment solutions (0 and 25 μM AlCl_3 in 0.4 mM CaCl_2 , pH 4.3) of Dade and Romano over (A) 0, 3, 6, and 12 h and (B) 1, 2, 3, and 4 d. A randomized experimental design was used with 2 cultivars, and 2 levels of AlCl_3 , and 3-4 timed harvests. Values represent the means $\pm$ SE of 3 or 5 replicates.

A

Experimental conditions	Total Al (μmol)	Monomeric Al (μmol)	Total citrate (μmol)	Ratio of citrate to total Al
25 μM AlCl_3 (0 h)	1.1 ± 0.00	0.4 ± 0.00	-	-
Dade + Al (3 h)	0.4 ± 0.02	0.2 ± 0.01	0.01 ± 0.008	0.01
Dade + Al (6 h)	0.3 ± 0.04	0.1 ± 0.00	0.02 ± 0.005	0.07
Dade + Al (12 h)	0.3 ± 0.09	0.1 ± 0.06	0.03 ± 0.006	0.13
Romano + Al (3 h)	0.4 ± 0.01	0.2 ± 0.02	0.02 ± 0.005	0.04
Romano + Al (6 h)	0.3 ± 0.02	0.1 ± 0.01	0.05 ± 0.042	0.16
Romano + Al (12 h)	0.2 ± 0.02	0.0 ± 0.00	0.04 ± 0.012	0.23

B

Experimental conditions	Total Al (μmol)	Monomeric Al (μmol)	Total citrate (μmol)	Ratio of citrate to total Al
25 μM AlCl_3 (0 h)	1.0 ± 0.00	0.4 ± 0.00	-	-
Dade + Al (1 d)	0.6 ± 0.04	0.1 ± 0.03	0.3 ± 0.05	0.57
Dade + Al (2 d)	0.8 ± 0.04	0.1 ± 0.00	0.5 ± 0.03	0.65
Dade + Al (3 d)	0.9 ± 0.02	0.1 ± 0.00	0.6 ± 0.05	0.70
Dade + Al (4 d)	0.7 ± 0.05	0.1 ± 0.00	0.6 ± 0.04	0.76
Romano + Al (1 d)	0.3 ± 0.04	0.1 ± 0.01	0.3 ± 0.04	0.75
Romano + Al (2 d)	0.3 ± 0.07	0.1 ± 0.00	0.2 ± 0.03	0.73
Romano + Al (3 d)	0.5 ± 0.11	0.1 ± 0.00	0.4 ± 0.07	0.83
Romano + Al (4 d)	0.4 ± 0.07	0.1 ± 0.00	0.3 ± 0.03	0.81

3.2.6. Examination of pH changes in treatment solutions

The pH of control and Al solutions was significantly different over both the 3-12 h and 1-4 d time periods. There were no significant differences in pH between Dade and Romano cultivars during the 3-12 h period. Conversely, the pH was significantly less in Dade treatment solutions over the 1-4 d time period. In both cultivars, the pH of all treatment solutions was ≥ 4.6 at all times (Table 4a, b).

3.3. Observation of Root Injury in Response to Aluminum in a High Volume System

3.3.1. Aluminum-induced root tip injury

Differential interference contrast light microscopy was used as a tool to observe primary root morphology and to further characterize Al injury in Dade and Romano plants (Figure 8, 9). Primary root tips of Romano plants grown under control conditions in pre-treatment solutions (0 d) showed no signs of Al injury. Cells of the outer cortex were regularly shaped and arranged in highly organized, linear files. After 1 d of exposure to 50 μM Al, cortical cells appeared swollen and misshapen, and alignment was slightly altered. After 2, 3, and 4 d of exposure, cortical cell damage worsened and cell files were no longer linear and organized. Damage was exacerbated after a 5 d exposure to 50 μM Al, and cell walls appeared highly irregular or non-existent in certain cells (Figure 8).

Primary root tips of Dade plants grown in pre-treatment solutions (0 d) also showed no signs of Al injury (Figure 9). Outer cells of the cortex appeared somewhat swollen and misshapen, and cell alignment was slightly altered after 1 d of exposure to 50 μM Al. Damage in Dade root tips appeared less serious than in those of Romano exposed to Al

Table 4. Determination of pH in treatment solutions in the low volume experimental system. Measurement of pH in treatment solutions (0 and 25 μM AlCl_3 in 0.4 mM CaCl_2 , pH 4.3) over (A) 3, 6, and 12 h and (B) 1, 2, 3, and 4 d. A randomized experimental design was used with 2 cultivars, and 2 levels of AlCl_3 , and 3-4 timed harvests. Values represent the means $\pm$ SE of five replicates.

A

Time (hours)	Dade (0 μM)	Dade (25 μM)	Romano (0 μM)	Romano (25 μM)
3	4.6 $\pm$ 0.03	4.5 $\pm$ 0.04	4.8 $\pm$ 0.15	4.5 $\pm$ 0.02
6	4.5 $\pm$ 0.05	4.7 $\pm$ 0.04	5.0 $\pm$ 0.03	4.6 $\pm$ 0.22
12	5.2 $\pm$ 0.07	4.9 $\pm$ 0.09	5.2 $\pm$ 0.04	5.0 $\pm$ 0.07

B

Time (days)	Dade (0 μM)	Dade (25 μM)	Romano (0 μM)	Romano (25 μM)
1	4.8 $\pm$ 0.07	4.9 $\pm$ 0.07	5.0 $\pm$ 0.04	5.4 $\pm$ 0.02
2	5.0 $\pm$ 0.19	4.9 $\pm$ 0.04	5.1 $\pm$ 0.00	5.5 $\pm$ 0.09
3	4.7 $\pm$ 0.10	4.9 $\pm$ 0.07	5.0 $\pm$ 0.07	5.1 $\pm$ 0.03
4	4.6 $\pm$ 0.20	4.7 $\pm$ 0.12	5.0 $\pm$ 0.10	5.2 $\pm$ 0.04



Figure 8. Aluminum injury in cortical cells of Romano over time. DIC images (40 X) of root tips of *Phaseolus vulgaris* L., Romano, before (A) 0 d, or after (B) 1, (C) 2, (D) 3, (E) 4, and (F) 5 d of exposure to 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3).

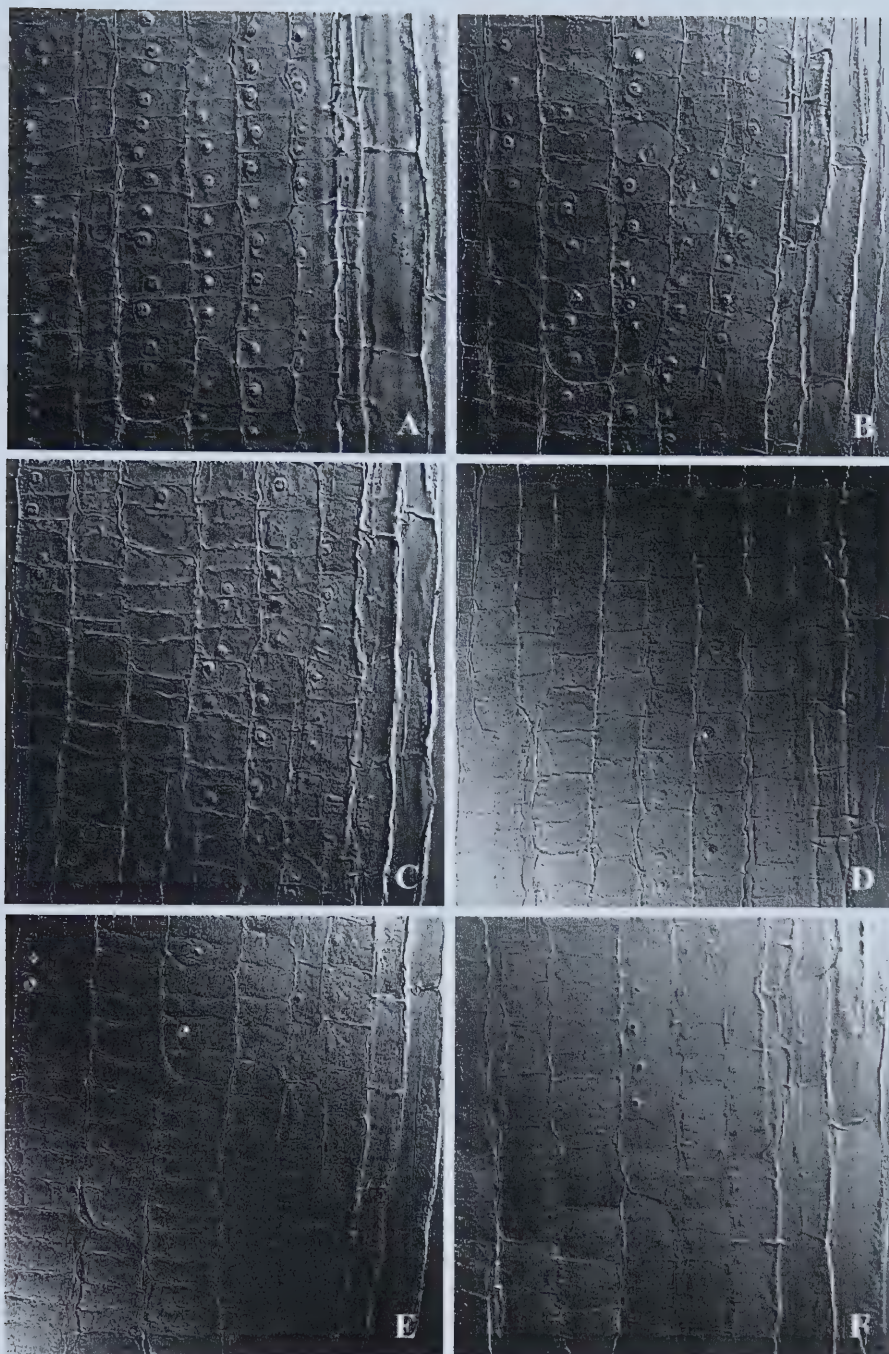


Figure 9. Aluminum injury in cortical cells of Dade over time. DIC images (40 X) of root tips of *Phaseolus vulgaris* L., Dade, before (A) 0 d or after (B) 1, (C) 2, (D) 3, (E) 4, and (F) 5 d of exposure to 50 μ M AlCl_3 in 10 L of full nutrient solution (pH 4.3).

for the same amount of time. Cortical cell damage was less visible and cell files were organized linearly after 2 d of Al treatment. Damage became even less visible after up to 5 d of exposure to 50 μ M Al, and cell walls generally appeared regular and properly aligned (Figure 9). Although nuclei were not always visible in the plane of focus of each image, they were present in most cortical cells. Similar observations of cortical cell morphology were made in all replicates examined.

3.3.2. Hematoxylin staining along the root

Dark purple hematoxylin staining occurred along the entire length of Romano roots exposed to 50 μ M Al solutions for 1-4 d. Dade roots exposed to Al solutions were only stained by a distinctive band of hematoxylin at each time point (Figure 10, 11, 12). This band of staining occurred in the area along the root that corresponded to the root tip region that was exposed to Al solutions upon initial planting at 0 d. These patterns of staining were seen in all 3 replicates of each cultivar stained with hematoxylin.

3.3.3. Observation of aluminum injury along the entire length of the root

The entire length of the primary root was examined using differential interference contrast light microscopy to determine if roots were damaged at some point during their exposure to Al solutions (Figure 13, 14, 15, Appendix). Outer cortical cells along the first 15.75 cm from the tip of Dade roots exposed to either 0 or 50 μ M Al solutions for 4 d were similar and showed no signs of Al damage (Figure 13, Appendix). However, cortical cells located 15.75 to 16.5 cm from the root tip showed considerable Al injury after a 4 d exposure to 50 μ M Al. Cells appeared swollen, cell walls were distorted, and

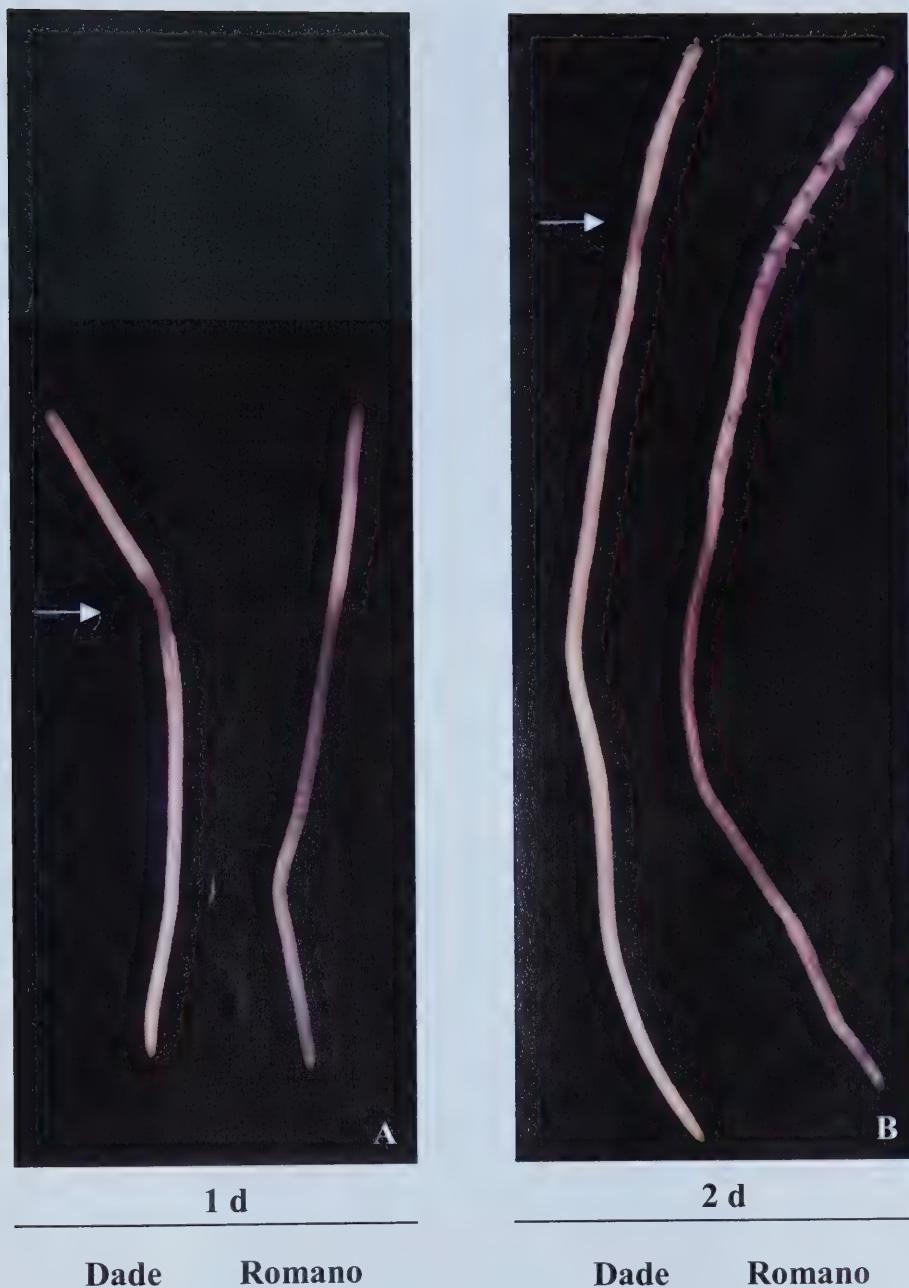


Figure 10. Hematoxylin staining of *Phaseolus vulgaris* L. exposed to aluminum. Images of Dade and Romano plants exposed to 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) for (A) 1 and (B) 2 days. Entire Romano roots became stained while only a distinct band of staining (arrows) occurred in Dade roots.

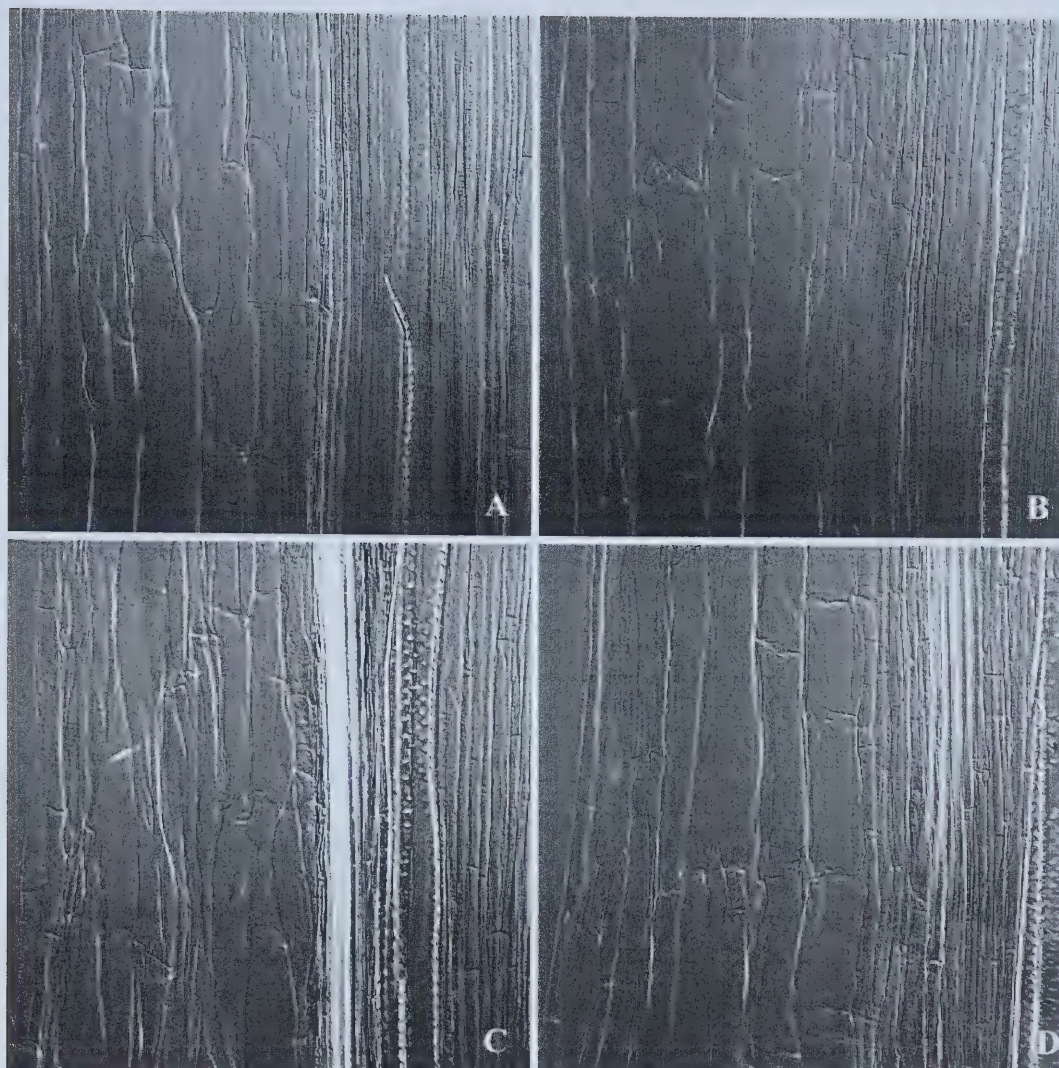


Figure 11. Hematoxylin staining of *Phaseolus vulgaris* L., Dade, exposed to aluminum. Images of Dade plants exposed to 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) for (A) 3, (B) 4, and (C) 5 days. Only a distinct band of purple staining occurred in Dade roots at each time point.



Figure 12. Hematoxylin staining of Dade and Romano root tips exposed to aluminum. Images of (A) Dade and (B) Romano root tips exposed to 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) for 5 days. Romano root tips became darkly stained while hematoxylin did not stain Dade root tips.

cortical cell alignment was disorganized (Figure 14). The location of this cortical cell injury corresponded to both the region of the root that became stained by hematoxylin and the root tip region that was first exposed to Al solutions upon planting at 0 d. Aluminum injury in cortical cells of Romano only occurred in the tip region of roots (Figure 15). Roots of cv. Romano had ceased to elongate in the presence of Al, thus observations were only available for a total of 8.25 cm. These observations of cortical cell morphology along the primary root were noted in all replicates examined.



0

50

Aluminum concentration (μM)

Figure 13. Cortical cells of primary roots of Dade unaffected by aluminum exposure. DIC images (16 X magnification) of cortical cells (A and B) 3.75 cm, and (C and D) 11.75 cm from the root tip of *Phaseolus vulgaris* L., Dade, exposed to either (A and C) 0 μM or (B and D) 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) for 4 days.

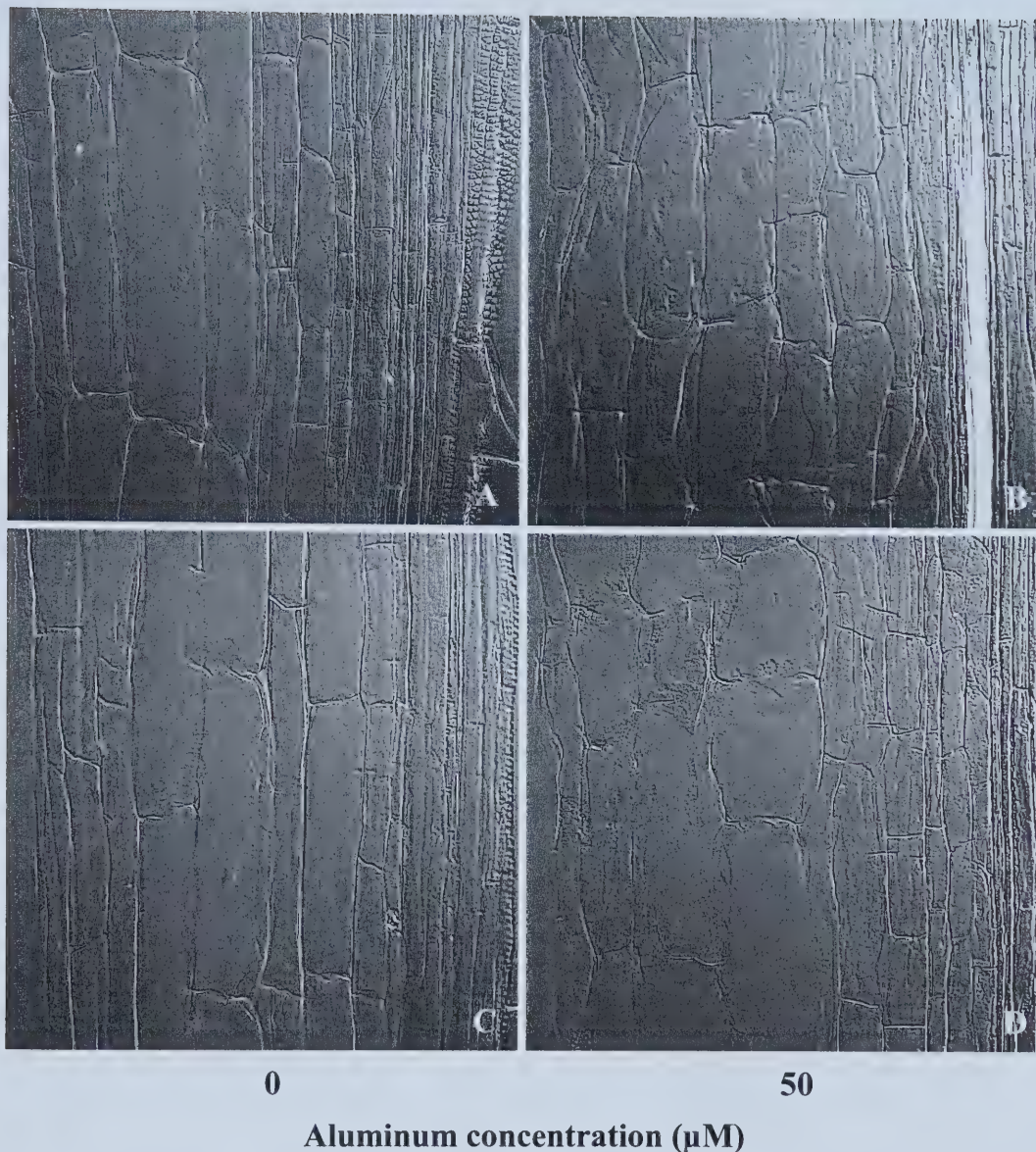


Figure 14. Aluminum injury in cortical cells of primary roots of Dade. DIC images (16 X magnification) of cortical cells (A and B) 15.75 cm, and (C and D) 16.50 cm from the root tip of *Phaseolus vulgaris* L., Dade, exposed to either (A and C) 0 μM or (B and D) 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) for 4 days.

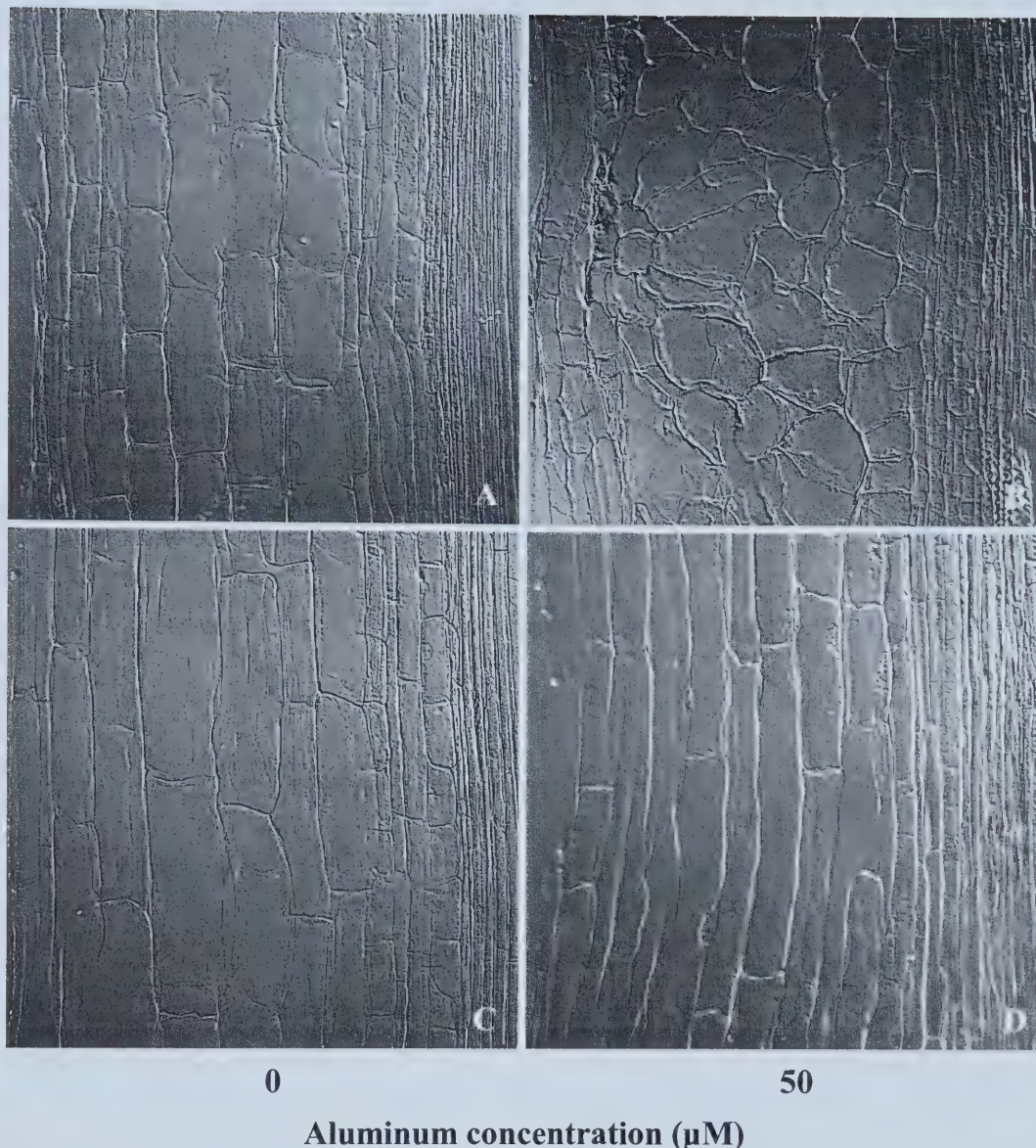


Figure 15. Aluminum injury in cortical cells of primary roots of Romano. DIC images (16 X magnification) of cortical cells (A and B) 0.75 cm, and (C and D) 8.25 cm from the root tip of *Phaseolus vulgaris* L., Romano, exposed to either (A and C) 0 μM or (B and D) 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) for 4 days.

4. DISCUSSION

Phaseolus vulgaris L. cultivars Dade (resistant) and Romano (sensitive) are differentially resistant to Al, and it has been proposed that resistance is induced in Dade (Foy *et al.*, 1967, 1972; Cumming *et al.*, 1992). It has also been shown that Dade exudes 10 fold more citrate than Romano in response to Al (Miyasaka *et al.*, 1991). Scientists have hypothesized that organic anions are involved in exclusion mechanisms of Al resistance. Thus, it would be interesting to determine if stimulation of citrate exudation in Dade is associated with the onset of Al-induced resistance. Aluminum-citrate complexes have a high stability constant (12.26 ± 0.28) and a greater ability to detoxify Al than other Al^{3+} -organic anion complexes (Hue *et al.*, 1986). In addition, studies have shown that exogenous addition of citrate promotes recovery of plant roots after Al exposure (Ownby and Popham, 1989). Differential resistance to Al and the exudation of a strong chelator (citrate) in relatively large amounts makes snapbean an ideal experimental species to examine the induction of Al resistance, and to further characterize root response to Al over the period of induction.

4.1. Analysis of Plant Response to Aluminum in a High Volume System

4.1.1. Differential resistance to aluminum exists between Dade and Romano

Previous soil and hydroponic studies indicated that snapbean cultivars Dade and Romano were differentially resistant to Al (Foy *et al.*, 1967, 1972). My long-term (16 d) dose response experiments in a high volume system confirmed that roots and shoots of Dade were significantly more resistant than those of Romano over a range of Al concentrations.

In cv. Romano, both shoot and root dry mass declined upon exposure to 10 μM Al. In cv. Dade, dry mass of roots declined upon exposure to Al concentrations greater than 40 μM , while shoot dry mass was adversely affected by Al concentrations exceeding 100 μM (Figure 1a, b). Foy *et al.* (1972) also described a more harmful effect of Al on root growth. Romano roots began showing Al toxicity symptoms at concentrations exceeding 10 μM Al, while 200 μM Al was required to have the same effect on Dade (Figure 1, 2). Roots showing toxicity symptoms were brown, brittle, and stunted, with limited lateral root growth (Figure 2).

Researchers generally agree that Al^{3+} and Al_{13} are toxic to plant growth (Kinraide, 1997). Solution pH was adjusted to 4.3 every other day to encourage consistent experimental conditions and to promote the presence of free Al^{3+} in nutrient solutions, as Al speciation is influenced by many variables (see Introduction). These adjustments were also made to ensure that differential resistance was not affected by differential pH changes in nutrient solutions by the two cultivars. In their studies, Foy *et al.* (1972) and Miyasaka *et al.* (1991) did not attempt to control solution pH over time. Both groups determined that differential resistance between Dade and Romano was attributed to Al rather than to differential resistance to acidity, because final pH measurements in Al solutions of both cultivars were essentially the same. In light of the precautions taken to keep solution pH consistent over time and the results from past studies, differential resistance between Dade and Romano was likely attributed to Al in the current study.

4.1.2. Aluminum resistance in Dade is induced over time

In a previous study, Cumming *et al.* (1992) suggested that Al resistance in Dade was an acquired or induced trait. Although Dade was more resistant than Romano to 10 μM Al over 21 d, they observed that root elongation in Dade and Romano was inhibited to a similar extent after 8 h of exposure. Dade roots subsequently recovered after 1-2 d of Al exposure. These differences were only apparent when the data were expressed as percent of control values over time. Root elongation (mm) appeared to be similarly inhibited in both cultivars at all Al concentrations in a 72 h dose response (0-120 μM Al) experiment.

A time course of primary root response to Al was repeated in an effort to verify the hypothesis of Al-induced resistance in Dade. In previous long-term dose response assessments, the greatest difference in root response between Dade and Romano existed when plants were exposed to 40-100 μM Al (Figure 1), therefore an Al concentration of 50 μM AlCl_3 was used in time course experiments. Inhibition of root elongation in response to Al stress is easily and rapidly observed (Clarkson, 1965; Foy *et al.*, 1978; Taylor, 1989; Kochian, 1995), thus inhibition of elongation was used as a marker of Al injury. Aluminum treatment inhibited primary root elongation to a similar degree in cultivars Dade and Romano over the 3-24 h period (Figure 3a). The linear trend of primary root elongation in Dade and Romano exposed to 50 μM Al was significantly different from roots in control solutions over this time period. However, root elongation in cv. Dade increased over time and recovered to levels similar to controls after 5 d of Al exposure. Roots of cv. Romano remained sensitive to Al over the entire treatment period.

Primary roots of Dade elongated 3 fold more than Romano roots after 5 d of Al exposure (Figure 3a, b).

The pH of treatment solutions was measured daily before readjustment to examine pH fluctuations over time. Seedlings of cv. Romano grown under control conditions altered pH significantly more than seedlings of cv. Dade from 3-5 d (Table 2). Fleming (1983) showed that pH changes in nutrient solutions were related to the source and supply of nitrogen present, and determined that varietal disparities in daily pH fluctuations were due to differences in NO_3^- and NH_4^+ uptake. Wheat varieties that quickly depleted nutrient solutions of NH_4^+ caused pH to decline more rapidly. It is unlikely that plant-induced pH changes contributed to the differential resistance between Dade and Romano, as significant pH differences did not occur between the two cultivars grown in Al solutions at any time. In addition, pH in Al solutions never rose above 4.4 in the 24 h period prior to readjustment (Table 2). Moreover, tests for monomeric Al confirmed that depletion did not occur over time and that a soluble, monomeric form of Al was present in treatment solutions throughout the entire treatment period (Table 1).

These time course experiments demonstrated that differential resistance between Dade and Romano was only apparent after 1 to 2 d of exposure to 50 μM Al, suggesting that Al resistance was induced in Dade. Others have suggested that induction of Al resistance occurs in several species (Jorge and Arruda, 1997; Vázquez *et al.*, 1999; Gunsé *et al.*, 2000; Ma *et al.*, 2000). As was the case with the Cumming *et al.* (1992) study, the induction of Al resistance reported in these experiments was not always clear. For

example, Jorge and Arruda (1997) correlated the reduction of hematoxylin staining of root tips over time with a recovery of root growth, and increased exudation of organic anions in a resistant cultivar of maize. Upon closer examination of their data, it is evident that relative root growth in the resistant variety of maize declined during the first 20 h of Al exposure, and then continued at a constant rate over the next 100 h. Root growth (mm) and rates of relative root growth never recovered to control levels over time. In addition, there was no clear temporal relationship between the onset of steady state root growth, the reduction in hematoxylin staining, and exudation of organic anions.

Gunsé *et al.* (2000) reported recovery of root elongation, reduced callose production in cortex cells, and decreased staining of Al by morin, in root tips of Al-resistant maize after a lag period of more than 4 h. Extended time course experiments were not undertaken to determine exactly when induction occurred and if resistance was transient or not. These authors proposed that a change in the speciation of Al in root tips was responsible for this recovery, but did not test this hypothesis any further. Vázquez *et al.* (1999) also reported a recovery in root elongation in an Al-resistant maize variety after 24 h of Al exposure. Unfortunately, these authors did not examine root response over an extended time and did not make any comparisons to a sensitive counterpart. In addition, a previous study examining this same Al-resistant variety of maize determined that recovery from short-term inhibition of root elongation was a result of amelioration of proton toxicity by Al (Llugany *et al.*, 1995).

On the other hand, Ma *et al.* (2000) showed a clear correlation in time between the exudation of organic anions and the recovery of Al-induced inhibition of root elongation in an Al-resistant line of triticale. Unfortunately, elongation of Al-treated roots never reached control levels. These authors suggested that Al-induced release of organic acids was responsible for the reduction in the inhibition of root elongation by Al. Yang *et al.* (2000) also made a similar claim regarding Al-induced resistance in soybean, but their data do not show a clear Al-induced inhibition and recovery of relative root elongation over time.

Although data from earlier studies support the hypothesis of Al-induced resistance in several plant species, the results are not entirely convincing. Results from time course experiments in the current study show that cultivars Dade and Romano are differentially resistant to Al after 1-2 d of exposure. Time course data also strengthen the Cumming *et al.* (1992) hypothesis that Al resistance is induced in Dade. However, stronger support would be provided if the Al-induced recovery in root elongation was correlated in time with similar changes in other markers of Al injury and/or with the induction of possible Al resistance mechanisms.

4.2. Analysis of Plant Response to Aluminum in a Low Volume System

A low volume (45 mL), aseptic culture system was developed to measure short-term responses of *Phaseolus vulgaris* L. to Al. To measure citrate exudation enzymatically, it was necessary to grow plants under specific conditions. Protocols for aseptic seed germination and seedling growth were developed and optimized as a sterile growth

system had to be maintained to avoid bacterial degradation of citrate. Plants were grown in simple salt solutions (0 or 25 μM AlCl_3 in 0.4 mM CaCl_2 , pH 4.3) and at a low plant to volume ratio (1 plant in 45 ml) to allow for sensitive measurement of root exudates over short time periods. Treatment solutions also had to be well aerated (rather than simply agitated on a shaker) for optimal growth of control plants. In addition, care was taken never to let seeds become immersed in solutions, as *Phaseolus vulgaris* L. seeds were sensitive to soaking injury (Davis, 1997; Pretorius *et al.*, 1998).

4.2.1. Citrate exudation is induced by aluminum in Dade

In their comparative study, Lee and Foy (1986) suggested that Dade (resistant) had a greater potential for chelating and detoxifying Al with organic anions than Romano (sensitive). Although malate and citrate levels in roots of both cultivars declined markedly in the presence of Al stress, they observed that roots of cv. Dade contained more organic anions in both the presence and absence of Al than cv. Romano. Several years later, Miyasaka *et al.* (1991) demonstrated that Dade released 10 times more citrate into the rhizosphere than Romano, and 70 times more citrate than controls in response to 8 d of Al exposure. This led to the hypothesis that citrate exudation was a mechanism of Al resistance in the snapbean cv. Dade.

To determine if organic anion exudation coincided with the timing of induction of Al resistance in Dade, citrate release was measured over time in a low volume system. Dade and Romano roots grown in both control and Al solutions exuded similar, low-level amounts of citrate over the first 3-12 h of treatment (Figure 4a). This lack of differential

citrate exudation was correlated with the inhibition of primary root elongation in Dade and Romano over the first 12 h of Al exposure in the high volume system (Figure 3a). With time (1-4 d), Al-treated plants of both cultivars exuded significantly greater amounts of citrate than those grown under control conditions (Figure 4b). Miyasaka *et al.* (1991) reported that Romano exuded only slightly more citrate than controls upon Al treatment. The discrepancy between the previous and present studies is likely due to differences in experimental conditions. In the Miyasaka *et al.* (1991) study, roots of cv. Romano were exposed to higher Al levels over a longer period of time (148 μM AlCl_3 over 8 d). Roots of cv. Romano would have been severely impaired, and metabolic processes would likely have been less active under those conditions.

Roots of cv. Dade released significantly more citrate than Romano roots after 1-4 d of exposure to 25 μM Al (Figure 4b). Interestingly, this differential exudation of citrate coincided with the appearance of differential resistance and the recovery of root growth in Dade in the high volume system (Figure 3b). In support of this finding, other studies have correlated the timing of induction of Al resistance in plants with the exudation of organic anions from roots, suggesting that an Al exclusion mechanism prevents entry of toxic Al into roots (Yang *et al.*, 2000; Ma *et al.*, 2000). For example, Ma *et al.* (2000) reported that Al inhibited root elongation in both resistant and sensitive lines of triticale during the first 12 h of treatment. Subsequently, as differential exudation of organic anions became evident, a recovery in root elongation was measured in the resistant line.

My data suggest that the Al-resistant cv. Dade follows the second pattern of organic anion efflux (see Introduction), and that a time lag of at least 24 h occurs between exposure to Al and organic anion exudation. These results support the idea that Al resistance is not constitutively expressed in Dade. Cumming *et al.* (1992) suggested that a stress-sensing mechanism was triggered in Dade upon exposure to Al, and that an initial decline in root elongation occurred until resistance mechanisms were activated. Olivetti *et al.* (1995) later reported that root cap cells of Dade and Romano differed in their electrical responses upon initial exposure to Al. They proposed that large, rapid depolarizations of the membrane potential of cells in Dade acted as an Al-induced signal that elicited metabolic responses allowing plants to acquire Al resistance. Olivetti *et al.* (1995) then suggested that the depolarizations may result from an Al-induced reduction of potassium channel conductance, plasma membrane proton pump activity, or opening of anion channels. Regardless of the signal responsible for induction of resistance mechanisms, recovery in elongation of Dade roots treated with Al and the corresponding rise in citrate exudation support the hypotheses that resistance is induced in Dade and that organic anion efflux is associated with Al resistance.

4.2.2. Effect of aluminum on root callose content over time

Deposition of callose in response to Al was used as another marker to verify that Al resistance was induced in Dade over time. Synthesis of 1,3- β -D-glucan (callose) is induced by Al mainly in the epidermis and in cells of the outer cortex of the root tip (Wissemeier *et al.*, 1987; Sivaguru *et al.*, 1999a), and has been used as a rapid and sensitive indicator of Al injury in many species (Wissemeier *et al.*, 1987, 1992; Llugany

et al., 1994; Schreiner *et al.*, 1994; Zhang *et al.*, 1994; Wissemeier and Horst, 1995; Sivaguru and Horst, 1998; Massot *et al.*, 1999; Kollmeier *et al.*, 2000). Both Dade and Romano produced up to 10 fold more callose than controls in the first 3-12 h of Al stress (Figure 7a). Similar results were obtained in *Phaseolus vulgaris* L. cell cultures exposed to a range of Al concentrations for 2 h (Taylor, 1995). As was the case with primary root responses in the high volume system (Figure 3a) and citrate measurements in the low volume system (Figure 4a), callose measurements demonstrated that both cultivars were similarly stressed by Al from 3-12 h. Dade appeared to be less sensitive to Al following 1 d of Al exposure, as roots only produced 4 times as much callose as controls. Dade roots treated with Al produced similar amounts of callose as controls beyond this time point, suggesting that they were no longer sensitive to Al (Figure 7b). Once again, the timing of the recovery of Dade roots from Al stress supports the theory of induction of Al resistance.

I expected that roots of the Al-sensitive cv. Romano would continue to produce high levels of callose in response to Al over the entire experimental period. However, roots of cv. Romano also appeared to be less sensitive to Al after 1 d of treatment (Figure 7b). These unexpected results could suggest that callose deposition may not be a suitable marker of Al stress in these snapbean cultivars. Earlier work by Larsen *et al.* (1996) showed that there was no correlation between callose deposition and Al-induced reduction in root growth in Al-sensitive *Arabidopsis* mutants. On the other hand, Massot *et al.* (1999) determined that callose production was a useful marker of whole plant resistance in various *Phaseolus vulgaris* L. cultivars exposed to 24 h of Al stress.

Unfortunately, cultivars Dade and Romano were not included in this investigation. It is also possible that the callose assay used in the current study was inappropriate for measurements beyond 24 h. Callose synthesis can also be measured via radioactive labeling (Zhang *et al.*, 1994), or visualized in root tips using various microscopic techniques (Northcote *et al.*, 1989; Schreiner *et al.*, 1994; Larsen *et al.*, 1998). Finally, it is also possible that the callose assay was giving a reliable measure of Al-induced injury and that the unexpected outcome of the callose time course experiments may signal problems inherent in the use of low volume systems.

4.2.3. Differential response to aluminum cannot be measured in a low volume system

To clarify the unexpected results obtained in callose experiments, the primary root response of plants grown in the low volume system was compared to that in the high volume system. Roots of both Dade and Romano plants were sensitive to Al from 3-12 h in the low volume system (Figure 5a). As was the case in the high volume system, no evidence of differential resistance existed between Dade and Romano up to this point.

Although induction of resistance and recovery of root elongation were evident in cv. Dade after 1 d of Al exposure in the low volume system (Figure 5b), plants did not recover from Al stress as quickly in this experimental system. Likewise, primary root elongation of Romano plants treated with Al was initially inhibited more severely in the low volume system. For example, Romano roots showed 31% of control elongation after 1 d of Al exposure in the low volume system (Figure 5b). In contrast, roots showed 44%

of control elongation after 1 d of Al exposure in the high volume system (Figure 3b). In support of this finding, an earlier study demonstrated that the recovery of Dade roots from Al stress was slower in simple salt solutions than in full nutrient solutions (Miyasaka and Hawes, 2001). This likely reflects the fact that Al injury to root tips was greater upon initial exposure, as more free Al^{3+} was available in simple salt solutions. Interaction of Al with other elements in full nutrient solutions of the high volume system will limit its potential toxic effect. Free Al^{3+} in Al solutions was predicted (GEOCHEM-PC, version 2.0) to be 14.5 μM in the low volume system and 9.6 μM in the high volume system.

Contrary to what was seen in the high volume system, Al-treated Romano roots also showed recovery in elongation following 2 d of treatment (Figure 5b). In addition, new tip growth in Romano roots did not exhibit typical Al injury symptoms (Figure 6). This pattern of recovery of root elongation was consistent with the decline of Al-induced callose deposition in Romano roots, where recovery was also evident on the third day of Al treatment. Thus, a major difference between the low and high volume systems was the fact that both cultivars recovered from Al injury in the low volume system. It is worth pointing out that Miyasaka and Hawes (2001) observed that relative root elongation of Dade and Romano plants grown in a higher volume (800 mL) of simple salt solutions was similar within the first 24 h of exposure to Al. Roots of cv. Dade continued to elongate over time and differential resistance was evident after 72 h.

4.2.4. Aluminum is depleted in solutions of the low volume system

The resumption of primary root elongation and the decline in callose deposition in roots of both Dade and Romano suggested that Al stress may no longer be present in the low volume system after 2 or 3 d. To test this hypothesis, total and monomeric Al levels were measured in the low volume experimental solutions over time. Total Al measurements reflected soluble Al and Al bound to slowly-exchanging ligands in solutions, while monomeric Al measurements accounted for only the soluble, monomeric species of Al.

There was significantly more total Al in low volume Al solutions (25 μM) than in control solutions over the 3-12 h and 1-4 d experimental periods, even though there was a substantial decline in the total Al present in solutions 3 h after planting. While Al solutions of both cultivars contained similar levels of total Al over 3-12 h, Al solutions of Dade contained significantly more total Al than those of Romano from 1-4 d (Table 3). These differences in total Al between Dade and Romano suggest that Al may enter roots more readily in Romano, but Al levels in root tissues were not measured in this study. In support of this hypothesis, earlier studies show differential accumulation of Al in root tissues, specifically in the epidermis and outer cortex of sensitive and resistant lines of wheat (Delhaize *et al.*, 1993a; Ownby, 1993). This phenomenon may be partly attributed to the differential exudation of citrate by Dade and Romano. Theoretically, more Al should be bound externally by citrate in Dade solutions, preventing Al entry into the root.

Total Al in solutions rose after 1 d of exposure to 25 μM Al in Dade, and on the third day of treatment in Romano (Table 3b). This increase may have been due to the rising levels

of citrate being exuded in response to Al exposure. Citrate is commonly used as a desorbing agent to remove exchangeable Al from cell walls (Zhang and Taylor, 1989; Archambault *et al.*, 1996; Taylor *et al.*, 2000). It is possible that Al was desorbed from root cell walls back into solution by citrate. The timing of this increase corresponded with differences in citrate release between Dade and Romano. Total Al rose in Romano solutions on the third day of Al treatment. Coincidentally, it was at this point that Romano roots released levels of citrate similar to those exuded by Dade at 1-2 d, when total Al levels began increasing in Dade treatment solutions (Table 3b).

Total Al measurements revealed that Al was still present in the 25 μ M Al solutions over time, but these values did not give an indication of levels of soluble, monomeric Al in solutions. Monomeric Al in 25 μ M Al solutions of Dade and Romano declined by 70% from 0-6 h. Although Al solutions of Dade and Romano still had significantly more Al than control solutions from 3-12 h, little toxic Al remained in treatment solutions after 1 d (Table 3). The absence of toxic Al in treatment solutions could explain the recovery in root elongation in cv. Romano over time. The disappearance of toxic Al from solutions after 1 d may be partly attributed to Al entry into the plant. In addition, levels of monomeric Al did not reflect the increasing pattern of total Al in solutions from 1-4 d, indicating that Al being added back into solutions was not a soluble, monomeric species. Factors that may have contributed to the lack of monomeric Al in the low volume system after 1 d are increasing citrate exudation, pH changes, precipitation of Al out of solutions, and Al accumulation by plants.

Citrate normally binds metals at a 1:1 molar ratio of ligand to metal (Kraschwitz and Howe-Grant, 1991; Harris *et al.*, 1996). One can get an indication of the proportion of Al that will potentially be bound by organic anions by comparing the levels of total Al and organic anions in treatment solutions. These ratios can be used to estimate the impact of organic anions on levels of toxic Al in solutions. Caution must be exercised in doing so, as Al speciation and solution variables such as pH and ionic strength will also affect the binding of Al to organic anions. Nonetheless, it was evident upon examining citrate:total Al that roots of Dade and Romano were not releasing enough citrate to bind total Al in solutions at a 1:1 ratio, even at the end of the 4 d experimental period (Table 3b). This suggests that the depletion of potentially toxic Al in treatment solutions was not solely due to chelation of Al by citrate, as Al solutions were already depleted after 1 d. In reality, the relationship between Al and citrate in solutions was likely more complex. If citrate exudation is indeed an Al-induced resistance mechanism, it is possible that the depletion of Al from treatment solutions itself prevented further increases in citrate exudation from roots of both Dade and Romano after 2 d of treatment (Figure 4). Moreover, several other solution variables changed dramatically over time in the low volume system.

It is known that the speciation of Al is altered by solution pH. A $\text{pH} < 4.5$ is thought to be required for Al to remain in the phytotoxic Al^{3+} form. Theoretically, a change of 0.1 pH units may result in a considerable decrease of toxic Al in solutions (Kinraide, 1991, 1997). In the low volume system, the pH of treatment solutions rose to $\text{pH} \geq 4.5$ at all times. In addition, cv. Romano induced a significantly higher solution pH than cv. Dade

solutions from 1-4 d (Table 4). This pH increase was correlated to the recovery of Romano roots from Al injury over this time period. It is probable that pH changes influenced the depletion of monomeric Al in treatment solutions over time.

The aseptic nature of the low volume system made it difficult to adjust solutions back to pH 4.3 daily without compromising sterility. It was also impractical to change solutions hourly or daily. The large volumes of pooled citrate samples resulting would have taken days to evaporate in preparation for citrate measurement. This raised a concern that losses of citrate would result from prolonged exposure to high temperatures and/or microbial degradation during this extended process. It may have been possible to use higher solution volumes to get better pH control, but Miyasaka and Hawes (2001) reported that pH still ranged from 4.6 to 4.9 in a system where 1 plant was grown in 800 mL of a simple salt solution for 96 h. Moreover, the use of higher volumes would likely make it impossible to get sensitive enzymatic citrate measurements over the short-term (hours). An attempt was made to control pH fluctuations in solutions by adding a potassium hydrogen phthalate buffer to solutions. Unfortunately, the concentration of buffer required made it impossible to achieve pH control without adversely affecting root growth.

It appears that the low volume system was not well suited for longer-term studies (≥ 24 h). It is likely that citrate exudation and pH fluctuations contributed to the depletion of monomeric Al in treatment solutions. Precipitation of Al in treatment solutions, Al entry into roots, and binding of Al to root surfaces may have also enhanced Al depletion, but the effect of these variables was not investigated. The recovery of root elongation and

decline in callose production in the low volume system was chronologically related to the induction of a possible Al resistance mechanism, citrate exudation, in cv. Dade over time. Unfortunately, as a result of Al depletion in the low volume system, plants of both Dade and Romano no longer displayed sensitive responses to Al after 24 h. Thus, I cannot be certain that the correlation between increased citrate exudation in Dade and recovery from Al injury is relevant in situations where plants are exposed to true Al stress.

4.3. Cellular markers of root injury and hematoxylin staining confirm that aluminum resistance is induced in Dade

Plants were once again grown in the high volume experimental system to further characterize the induction of Al resistance in Dade. In this system, differential resistance to Al between Dade and Romano was observed (Figure 1), induction was apparent in Dade (Figure 3), potentially toxic Al was not significantly depleted (Table 1), and pH did not fluctuate over time (Table 2). Previous studies have demonstrated that the root apex is most sensitive to Al injury (Ryan *et al.*, 1993; Sivaguru and Horst, 1998; Kollmeier *et al.*, 2000), thus cellular injury in primary root tips was observed using differential interference contrast light microscopy.

As expected, injury was obvious in root tips of Al-sensitive Romano after 1 d of Al exposure, and this cellular injury became more pronounced with time. Aluminum injury was most evident in cortical cells. These cells were enlarged and misshapen, and their cell walls were distorted and appeared to deteriorate over time. In addition, cells were no longer arranged in orderly, linear files (Figure 8). Similar symptoms of Al injury have

been described at the cellular level in root tips. These include swelling, increased vacuolation, thickening and stiffening of cell walls, loss of integrity of the plasma membrane, and disorganization and disintegration of epidermal and cortical cells (Fleming and Foy, 1968; Keser *et al.*, 1975, 1977; Bennet *et al.*, 1985a; Hecht-Buchholz *et al.*, 1990; Wheeler *et al.*, 1992; Ikeda and Tadano, 1993; de Lima and Copeland, 1994; Wagatsuma *et al.*, 1995; Moon *et al.*, 1997; Vázquez *et al.*, 1999). X-ray microanalysis suggests that Al initially binds strongly and rapidly to the cell wall of cortical and epidermal cells (Delhaize *et al.*, 1993a; Marienfeld and Stelzer, 1993; Minocha *et al.*, 2001). Kataoka *et al.* (1997) proposed that the formation of strong bonds by Al in outer cortical cells inhibited cell elongation, resulting in the destruction of the epidermis and cortex. However, it is not known whether the primary effect of Al is on cell division or on cell elongation.

Injury of cortical cells was also observed in root tips of Dade after 1 d of Al exposure, but symptoms were never as severe as in Romano. In addition, injury of cortical cells of Dade root tips became less evident over time (Figure 9). The disappearance of injury in Dade coincided with the recovery of primary root elongation over time, providing additional support to the hypothesis that resistance to Al is induced in Dade. In an earlier study, Vázquez *et al.* (1999) also reported transient Al toxicity symptoms in cells of a resistant variety of maize. After 4 h of Al treatment, a reduction in the rate of root elongation was accompanied by a thickening of tangential cell walls in internal cortex cells, and root tips were intensely stained by hematoxylin when compared to controls.

Following 24 h of Al exposure, root elongation rates had recovered to control values, and ultrastructural alterations and hematoxylin staining in root tips were no longer observed.

Vázquez *et al.* (1999) suggested that resistance mechanisms that induced changes in the speciation of apoplastic Al were activated, resulting in the disappearance of Al toxicity symptoms after 24 h. However, not all Al toxicity symptoms had disappeared after 24 h. There was no evidence of a toxic effect of Al on the plane of cell division of internal cortex cells of root tips after 4 h of exposure, but abnormal divisions and irregular arrangement of these cells became apparent after 24 h. Observations were not continued beyond 24 h to determine if these Al toxicity symptoms were also transient. Contrary to their suggestion, Vázquez *et al.* (1999) did not observe a chronological relationship between recovery of root elongation rates and the disappearance of Al injury. As mentioned earlier, there is also some doubt as to whether the recovery in root elongation rates was related to the induction of Al-resistance mechanisms. A study by Llugany *et al.* (1995) showed that this same variety of maize was highly proton-sensitive at pH 4.3, and that a transient stimulation of elongation resulted from amelioration of proton toxicity by Al.

Hematoxylin staining and observation of cell morphology along the entire length of primary roots were undertaken to further investigate the Al-induced injury in cortical cells of Dade root tips over time. It was acknowledged that the apparent disappearance of injury in Al-treated root tips of Dade could simply be a reflection of continued root elongation. As a result of continuing root elongation, cells within the root apices of Dade

and Romano would not share the same history of exposure to Al. Individual cells in root apices of Dade would have a reduced exposure to Al as new cells were continually being produced. In comparison, individual cells in root apices of Romano would have experienced a more prolonged exposure to Al.

Hematoxylin staining is used as a rapid screening tool to classify plants according to their sensitivity to Al (Polle *et al.*, 1978; Delhaize *et al.*, 1993a; Ownby, 1993; Giaveno and Filho, 2000). Entire roots of Al-treated Romano were darkly stained by hematoxylin in the high volume experiments. On the other hand, only a small, but distinctive band of hematoxylin staining was evident in Dade roots (Figure 10, 11, 12). Moreover, this band of staining in Dade occurred in the area along the root that corresponded to the root tip region that was originally exposed to Al upon planting at 0 d. Once again, these staining patterns confirmed that differential resistance to Al exists between Dade and Romano. Ownby (1993) proposed that selective hematoxylin staining was a result of direct damage to root cell membranes by Al. Although this hypothesis was not tested, Ownby (1993) suggested that Al-induced injury leads to phosphorous (P) leakage into the cell wall region, resulting in the formation of P-Al precipitates that form complexes with hematoxylin.

Jorge and Arruda (1997) correlated the disappearance of hematoxylin staining of root tips over time with a recovery of relative root growth in a resistant cultivar of maize. In contrast to the present study, root tips of both Al-sensitive and Al-resistant plants were stained intensely by hematoxylin after 24 h of Al treatment. A reduction in staining was

then seen only in the resistant cultivar after 72 h. Jorge and Arruda (1997) suggested that this reduction in staining intensity was due to the expulsion of previously absorbed Al from root tips over time. This hypothesis was not tested. Furthermore, it is not known whether hematoxylin complexes all species of Al bound to root tissues, and the persistence of hematoxylin complexes over time has not been investigated. As was mentioned earlier, the induction of resistance was not clear in this study, as root growth rates did not recover to control values.

To determine if hematoxylin staining was correlated with cellular injury along the root, cortical cell morphology was observed along the entire length of roots exposed to Al for 4 d. In Romano, cortical cell injury was only evident in the tip region of roots (Figure 15). As the root apex is the primary site of Al toxicity, we would not have expected to observe any cortical cell injury in mature tissues upon initial exposure to Al. In addition, no new growth was occurring in Romano roots after 4 d of exposure to 50 μ M Al. Thus, we would expect Al-injury in this cultivar to be localized in physical proximity to the root tip.

Cultivar Dade showed a different pattern of cortical cell injury. Normal cortical cell morphology was observed at the root tip and up to 15.75 cm along the root in Dade plants grown in both control and Al treatment solutions (Figure 13, 14 Appendix). However, injury to cortical cells similar to that seen in Romano root tips was observed beyond this point. When compared with controls, cortical cells appeared swollen and distended, their cell walls were distorted, and their alignment was disordered (Figure 14). The location of

this injury corresponded with both the location of the distinctive band of hematoxylin staining and the region of root tissue representing the root tip that was first exposed to Al solutions upon planting. In effect, this region of the root provided a history of previous response (injury) to Al. These observations suggested that roots were able to acquire resistance despite the fact that root tips of Dade were injured upon initial contact with Al. Aluminum appeared to have a transient effect on roots and did not cause cell death in Dade. Roots continued to grow in the presence of Al and new tissues appeared white and healthy with minimal cortical cell damage.

Observations of cellular markers of root injury and hematoxylin staining patterns in the current study indicate that Al resistance was induced in the snapbean cv. Dade. The coincidence in time between recovery from Al-induced inhibition of primary root elongation, the disappearance of cortical cell injury, and patterns of hematoxylin staining provides strong evidence of an induced mechanism of Al resistance. Several authors have suggested that Al resistance was induced in other species, but their data were not entirely convincing. No other study has provided clear evidence of a chronological relationship in the disappearance of numerous markers of Al injury and Al resistance. In addition, plants did not always become fully resistant to Al following induction and injury symptoms did not always disappear completely. My new data on cellular injury provide strong evidence that Dade roots were injured upon initial treatment with Al, and this exposure induced molecular, biochemical, and/or physiological changes that resulted in the disappearance of injury and sustained growth despite continuous Al exposure.

5. CONCLUDING STATEMENT

My work supports the hypothesis that resistance to Al is induced in cv. Dade. My research provides data that demonstrate a temporal relationship between the recovery of Al-induced inhibition of root elongation, patterns of hematoxylin staining, and the disappearance of cell wall injury, independent of pH changes and Al depletion in nutrient solutions. This work presented strong evidence that regardless of initial damage, roots of cv. Dade were able to overcome injury and grow normally despite continued exposure to Al.

The timing of differential citrate exudation by cv. Dade in the low volume system also corresponded to the recovery of root elongation in the high volume system. Many studies have correlated the timing of induction of Al resistance in plants with the exudation of organic anions from roots, suggesting that an Al exclusion mechanism prevented entry of toxic Al into roots. Unfortunately, because of problems associated with the low volume system, the current study did not determine conclusively that citrate release was linked to the induction of Al resistance in cv. Dade. Future work should focus on achieving pH control and maintaining monomeric Al levels in a low volume system that allows for the measurement of citrate.

My research suggests that this snapbean system will be an excellent tool in future investigations addressing the role of Al-induced gene activation in Al resistance mechanisms. This model system could be used to characterize patterns of organic anion

efflux, elucidate mechanisms of organic anion transport, and further examine metabolism and growth in response to Al. Continued study of the responses of resistant plants to Al is required to further define mechanisms of Al toxicity and resistance in plants. A more complete understanding of these phenomena will aid in the development of Al-resistant crops that are able to achieve greater productivity on acid soils throughout the world.

6. LITERATURE CITED

- Anoop, V. M., Basu, U., McCammon, M. T., McAlister-Henn, L. and G. J. Taylor.** 2003. Modulation of Citrate Metabolism Alters Aluminum Tolerance in Brewer's Yeast and Transgenic Canola Overexpressing a Mitochondrial Citrate Synthase. *Plant Physiol.* 132: 2205-2217.
- Archambault, D.J., Zhang, G. and G.J. Taylor.** 1996. A comparison of the kinetics of aluminum (Al) uptake and distribution in roots of wheat (*Triticum aestivum*) using different aluminum sources. A revision of the operational definition of symplastic Al. *Physiol. Plant.* 98: 578-586.
- Bartlett, R.J. and D.C. Riego.** 1972. Effect of chelation on the toxicity of aluminum. *Plant Soil.* 37: 419-423.
- Basu, U., Godbold, D. and G.J. Taylor.** 1994. Aluminum resistance in *Triticum aestivum* associated with enhanced exudation of malate. *J. Plant Physiol.* 144: 747-753.
- Basu, U., Good, A.G. and G.J. Taylor.** 2001. Transgenic *Brassica napus* plants overexpressing aluminium-induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminium. *Plant Cell Environ.* 24: 1269-1278.
- Bennet, R.J., Breen, C.M. and M.V. Fey.** 1987. The effects aluminium on root cap function and root development in *Zea mays* L. *Envir. Exp. Bot.* 27: 91-104.
- Bennet, R.J., Breen, C.M. and M.V. Fey.** 1985a. Aluminium induced changes in the morphology of the quiescent centre, proximal meristem and growth region of the root of *Zea mays*. *S. Afr. J. Bot.* 51: 355-362.
- Bennet, R.J., Breen, C.M. and M.V. Fey.** 1985b. The primary site of aluminium injury in the root of *Zea mays* L. *S. Afr. J. Plant Soil.* 2: 8-17.
- Blancaflor, E.B., Jones, D.L., and S. Gilroy.** 1998. Alterations in the cytoskeleton accompany aluminum-induced growth inhibition and morphological changes in primary roots of maize. *Plant Physiol.* 118: 159-172.
- Cakmak, I. and W.J. Horst.** 1991. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). *Physiol. Plant.* 83: 463-468.
- Chang, Y.-C., Yamamoto, Y. and H. Matsumoto.** 1999. Accumulation of aluminium in the cell wall pectin in cultured tobacco (*Nicotiana tabacum* L.) cells treated with a combination of aluminium and iron. *Plant Cell Environ.* 22: 1009-1017.

- Clarkson, D.T.** 1965. The effect of aluminium and some other trivalent metal cations on cell division in the root apices of *Allium cepa*. *Ann. Bot.* 29: 309-315.
- Cumming, J.R., Cumming A.B. and G.J. Taylor.** 1992. Patterns of root respiration associated with the induction of aluminum tolerance in *Phaseolus vulgaris* L. *J. Exp. Bot.* 43: 1075-1081.
- Dagley, S.** 1974. Citrate: UV spectrophotometric determination. In Bergmeyer, H.U. (ed). *Methods of Enzymatic Analysis*, Vol. 3 (2nd ed). Academic Press, New York, pp. 1562-1565.
- Davis, J.H.C.** 1997. *Phaseolus* beans. In Wien, H.C. (ed). *The Physiology of Vegetable Crops*. CAB International. Oxford, pp. 409-428.
- de la Fuente, J.M., Ramirez-Rodríguez, V., Cabrera-Ponce, J.L. and L. Herrera-Estrella.** 1997. Aluminum tolerance in transgenic plants by alteration of citrate synthesis. *Science*. 276: 1566-1568.
- de Lima, M.L. and L. Copeland.** 1994. Changes in the ultrastructure of the root tip of wheat following exposure to aluminium. *Aust. J. Plant Physiol.* 21: 85-94.
- Delhaize, E., Craig, S., Beaton, C.D., Bennet, R.J., Jagadish, V.C. and P.J. Randall.** 1993a. Aluminum tolerance in wheat (*Triticum aestivum* L.) I. Uptake and distribution of aluminum in root apices. *Plant Physiol.* 103: 685-693.
- Delhaize, E., Hebb, D.M. and P.R. Ryan.** 2001. Expression of a *Pseudomonas aeruginosa* citrate synthase gene in tobacco is not associated with either enhanced citrate accumulation or efflux. *Plant Physiol.* 125: 2059-2067.
- Delhaize, E., Ryan, P.R., and P.J. Randall.** 1993b. Aluminum tolerance in wheat (*Triticum aestivum* L.) II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiol.* 103: 695-702.
- Delhaize, E. and P.R. Ryan.** 1995. Aluminum toxicity and tolerance in plants. *Plant Physiol.* 107: 315-321.
- Ezaki, B., Katsuhara, M., Kawamura, M. and H. Matsumoto.** 2001. Different mechanisms of four aluminum (Al)-resistant transgenes for Al toxicity in Arabidopsis. *Plant Physiol.* 127: 918-927.
- Fleming, A.L.** 1983. Ammonium uptake by wheat varieties differing in Al tolerance. *Agron. J.* 75: 726-730.
- Fleming, A.L. and C.D. Foy.** 1968. Root structure reflects differential aluminum tolerance in wheat varieties. *Agron. J.* 60: 172-176.

- Foy, C.D.** 1984. Physiological effects of hydrogen, aluminum, and manganese toxicities in acid soil. In *Soil Acidity and Liming – Agronomy monograph no.12* (2nd ed). ASA-CSSA-SSSA. Madison, pp. 57-97.
- Foy, C.D., Armiger, W.H., Fleming, A.L., and W.J. Zaumeyer.** 1967. Differential tolerance of dry bean, snapbean, and lima bean varieties to an acid soil high in exchangeable aluminum. *Agron. J.* 59: 561-563.
- Foy, C.D., Chaney, R.L., and M.C. White.** 1978. The physiology of metal toxicity in plants. *Ann. Rev. Plant Physiol.* 29: 511-566.
- Foy, C.D., Fleming, A.L. and G.C. Gerloff.** 1972. Differential aluminum tolerance in two snapbean varieties. *Agron. J.* 64: 815-818.
- Gallardo, F., Borie, F., Alvear, M. and E. von Baer.** 1999. Evaluation of aluminum tolerance of three barley cultivars by two short-term screening methods and field experiments. *Soil Sci. Plant Nutr.* 45: 713-719.
- Gaume, A., Mächler, F. and E. Frossard.** 2001. Aluminum resistance in two cultivars of *Zea mays* L.: Root exudation of organic acids and influence of phosphorus nutrition. *Plant Soil.* 234: 73-81.
- Giaveno, C.D. and J.B. Miranda Filho.** 2000. Rapid screening for aluminum tolerance in maize (*Zea mays* L.). *Genet. Mol. Biol.* 23: 847-850.
- Ginting, S., Johnson, B.B. and S.Wilkens.** 1998. Alleviation of aluminum phytotoxicity on soybean growth by organic anions in nutrient solutions. *Aus. J. Plant Physiol.* 25: 901-908.
- Gunsé, B., Poschenrieder, C. and J. Barceló.** 2000. The role of ethylene metabolism in the short-term responses to aluminum by roots of two maize cultivars different in Al-resistance. *Envir. Exp. Bot.* 43: 73-81.
- Gunsé, B., Poschenrieder, C., and J. Barcélo.** 1997. Water transport properties of roots and root cortical cells in proton- and Al-stressed maize varieties. *Plant Physiol.* 113: 595-602.
- Harris, W.R., Berthon, G., Day, J.P., Exley, C., Flaten, T.P., Forbes, W.F., Kiss, T., Orvig, C. and P.F. Zatta.** 1996. Speciation of aluminum in biological systems. *J. Toxicol. Environ. Health.* 48: 543-568.
- Haug, A.** 1984. Molecular aspects of aluminum toxicity. *CRC Crit. Rev. Plant Sci.* 1: 345-373.

- Hecht-Buchholz, Ch., Brady, D.J., Asher, C.J. and D.G. Edwards.** 1990. Effects of low activities of aluminium on soybean (*Glycine max*). II. Root cell structure and root hair development. In van Beusichem, M.L. (ed). Plant nutrition – physiology and applications. Kluwer Academic Publishers, Dordrecht. pp. 335-343.
- Horst, W.J.** 1995. The role of the apoplast in aluminium toxicity and resistance of higher plants: a review. *Z. Pflanzenernahr. Bodenk.* 158: 419-428.
- Horst, W.J., Asher, C.J., Cakmak, I., Szulkiewicz, P. and A.H. Wissemeier.** 1992. Short-term responses of soybean roots to aluminium. *J. Plant Physiol.* 140: 174-178.
- Hue, N.V., Craddock, G.R., and F. Adams.** 1986. Effect of organic acids on aluminum toxicity in subsoils. *Soil Sci. Soc. Am. J.* 50: 28-34.
- Ikeda, H. and T. Tadano.** 1993. Ultrastructural changes of the root tip cells in barley induced by a comparatively low concentration of aluminum. *Soil Sci. Plant Nutr.* 39: 109-117.
- Johansen, D.A.** 1940. *Plant Microtechnique*. McGraw-Hill Book Company, Inc., New York. 326 p.
- Johnson, P.A. and R.J. Bennet.** 1991. Aluminium tolerance of root cap cells. *J. Plant Physiol.* 137: 760-762.
- Jones, D.L.** 1998. Organic acids in the rhizosphere- a critical review. *Plant Soil.* 205: 25-44.
- Jones, D.L. and P.R. Darrah.** 1995. Influx and efflux of organic acids across the soil-root interface of *Zea mays* L. and its implications in rhizosphere C flow. *Plant Soil.* 173: 103-109.
- Jorge, R.A. and P. Arruda.** 1997. Aluminum-induced organic acid exudation by roots of an aluminum-tolerant tropical maize. *Phytochemistry.* 45: 675-681.
- Kataoka, T., Mori, M., Nakanishi, T.M., Matsumoto, S. and A. Uchiumi.** 1997. Highly sensitive analytical method for aluminum movement in soybean root through lumogallion staining. *J. Plant Res.* 110: 305-309.
- Kerven, G.L., Edwards, D.G., Asher, C.J., Hallman, P.S. and S. Kokot.** 1989a. Aluminum determination in soil solution. I. Evaluation of existing colorimetric and separation methods for the determination of inorganic monomeric aluminium in the presence of organic acid ligands. *Aust. J. Soil Res.* 27: 79-90.

- Kerven, G.L., Edwards, D.G., Asher, C.J., Hallman, P.S. and S. Kokot.** 1989b. Aluminum determination in soil solution. II. Short-term colorimetric procedures for the measurement of inorganic monomeric aluminium in the presence of organic acid ligands. *Aust. J. Soil Res.* 27: 91-102.
- Keser, M., Neubauer, B.F. and F.E. Hutchinson.** 1975. Influence of aluminum ions on developmental morphology of sugarbeet roots. *Agron. J.* 67: 84-88.
- Keser, M., Neubauer, B.F., Hutchinson, F.E. and D.B. Verrill.** 1977. Differential aluminum tolerance of sugarbeet cultivars, as evidenced by anatomical structure. *Agron. J.* 69: 347-350.
- Kinraide, T.B.** 1991. Identity of the rhizotoxic aluminum species. *Plant Soil.* 134: 167-178.
- Kinraide, T.B.** 1997. Reconsidering the rhizotoxicity of hydroxyl, sulphate, and fluoride complexes of aluminum. *J. Exp. Bot.* 48: 1115-1124.
- Kinraide, T.B. and D.R. Parker.** 1990. Apparent phytotoxicity of mononuclear hydroxy-aluminum to four dicotyledonous species. *Physiol. Plant.* 79: 283-288.
- Kochian, L.V.** 1995. Cellular mechanisms of aluminum toxicity and resistance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46: 237-260.
- Kohle, H., Jeblick, W., Poten, F., Blaschek, W. and H. Kauss.** 1985. Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+} -dependent process. *Plant Physiol.* 77: 544-551.
- Kollmeier, M., Dietrich, P., Bauer, C.S., Horst, W.J. and R. Hedrich.** 2001. Aluminum activates a citrate-permeable anion channel in the aluminum sensitive zone of the maize root apex. A comparison between an aluminum-sensitive and an aluminum-resistant cultivar. *Plant Physiol.* 126: 397-410.
- Kollmeier, M., Felle, H.H. and W.J. Horst.** 2000. Genotypical differences in aluminum resistance of maize are expressed in the distal part of the transition zone. Is reduced basipetal auxin flow involved in inhibition of root elongation by aluminum? *Plant Physiol.* 122: 945-956.
- Koyama, H., Kawamura, A., Kihara, T., Hara, T., Takita, E. and D. Shibata.** 2000. Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* improved growth on a phosphorus limited soil. *Plant Cell Physiol.* 41:1030-1037.
- Koyama, H., Takita, E., Kawamura, A., Hara, T. and D. Shibata.** 1999. Over expression of mitochondrial citrate synthase gene improves the growth of carrot cells in Al-phosphate medium. *Plant Cell Physiol.* 40: 482-488.

- Kraschwitz, J.I. and M. Howe-Grant eds.** 1991. Citric acid. In: Kirk-Othmer encyclopedia of chemical technology: Vol. 6. 4th ed. Wiley-Interscience, New York. pp. 354-380.
- Larsen, P.B., Degenhardt, J., Tai, C.Y., Stenzler, L.M., Howell, S.H. and L.V. Kochian.** 1998. Aluminum-resistant Arabidopsis mutants that exhibit altered patterns of aluminum accumulation and organic acid release from roots. *Plant Physiol.* 117: 9-18.
- Larsen, P.B., Tai, C-Y., Kochian, L.V. and S.H. Howell.** 1996. Arabidopsis mutants with increased sensitivity to aluminum. *Plant Physiol.* 110: 743-751.
- Le Van, H., Kuraishi, S. and N. Sakurai.** 1994. Aluminum-induced rapid root inhibition and changes in cell-wall components of squash seedlings. *Plant Physiol.* 106: 971-976.
- Lee, E.H. and C.D. Foy.** 1986. Aluminum tolerances of two snapbean cultivars related to organic acid content evaluated by high-performance liquid chromatography. *J. Plant Nutr.* 9: 1481-1498.
- Li, X.F., Ma J.F. and H. Matsumoto.** 2002. Aluminum-induced secretion of both citrate and malate in rye. *Plant Soil.* 242: 235-243.
- Li, X.F., Ma J.F. and H. Matsumoto.** 2000. Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.* 123: 1537-1543.
- Llugany, M., Massot, N., Wissemeier, A.H., Poschenrieder, C., Horst, W.J. and J. Barceló.** 1994. Aluminium tolerance of maize cultivars as assessed by callose production and root elongation. *Z. Pflanzenernahr. Bodenk.* 157: 447-451.
- Llugany, M., Poschenrieder, C. and J. Barceló.** 1995. Monitoring of aluminium-induced inhibition of root elongation in four maize cultivars differing in tolerance to aluminium and proton toxicity. *Physiol. Plant.* 93: 265-271.
- Luo, H.M., Watanabe, T., Shinano, T. and T. Tadano.** 1999. Comparison of aluminum tolerance and phosphate absorption between Rape (*Brassica napus* L.) and Tomato (*Lycopersicum esculentum* Mill.) in relation to organic acid exudation. *Soil Sci. Plant Nutr.* 45: 897-907.
- Ma, J.F.** 1997. Detoxifying aluminium in buckwheat. *Nature.* 390: 569-570.
- Ma, J.F.** 2000. Role of organic acids in detoxification of aluminum in higher plants. *Plant Cell Physiol.* 41: 383-390.
- Ma, J.F., Ryan, P.R. and E. Delhaize.** 2001. Aluminum tolerance in plants and the complexing role of organic acids. *Trends in Plant Science.* 6: 273-278.

- Ma, J.F., Taketa, S. and Z.M. Yang.** 2000. Aluminum tolerance genes on the short arm of chromosome 3R are linked to organic acid release in Triticale. *Plant Physiol.* 122: 687-694.
- Ma, J.F., Zheng, S.J. and H. Matsumoto.** 1997a. Specific secretion of citric acid induced by Al stress in *Cassia tora* L. *Plant Cell Physiol.* 38: 1019-1025.
- Ma, J.F., Zheng, S.J., Matsumoto, H. and S. Hiradate.** 1997b. Detoxifying aluminium with buckwheat. *Nature.* 390: 569-570.
- Ma, Z., and S.C. Miyasaka.** 1998. Oxalate exudation by taro in response to Al. *Plant Physiol.* 118: 861-865.
- Marienfeld, S. and R. Stelzer.** 1993. X-ray microanalyses in roots of Al-treated *Avena sativa* plants. *J. Plant Physiol.* 141: 569-573.
- Marschner, H.** 1995. Mineral Nutrition of Higher Plants (2nd ed). Academic Press Inc., London, 889 p.
- Martin, R.B.** 1988. Bioinorganic chemistry of aluminum. In Sigel, H and A. Sigel (eds). Metal Ions In Biological Systems, Volume 24 Aluminum and its role in biology. Marcel Dekker, Inc. New York, pp. 1-57.
- Massot, N., Llugany, M., Poschenrieder, C. and J. Barceló.** 1999. Callose production as indicator of aluminum toxicity in bean cultivars. *J. Plant Nutr.* 22: 1-10.
- Matsumoto, H.** 1991. Biochemical mechanism of the toxicity of aluminium and the sequestration of aluminium in plant cells. In Wright, R.J., Baligar, V.C. and R.P. Murrmann (eds). Plant-soil interactions at low pH. Proceedings of the second international symposium on plant-soil interactions at low pH, 24-29 June 1990, Beckley, West Virginia, USA. Kluwer Academic Publishers, Dordrecht. pp. 825-838.
- Matsumoto, H.** 2000. Cell biology of aluminum toxicity and tolerance in higher plants. *Int. Rev. Cytol.* 200: 1-46.
- Menzies, N.W., Kerven, G.L., Bell, L.C. and D.G. Edwards.** 1992. Determination of total soluble aluminum in soil solution using pyrocatechol violet, lanthanum and iron to discriminate against micro-particulates and organic ligands. *Commun. Soil Sci. Plant Anal.* 23: 2525-2545.
- Minocha, R., Mc Quattie, C., Fagerberg, W., Long, S. and E.W. Noh.** 2001. Effects of aluminum in red spruce (*Picea rubens*) cell cultures: Cell growth and viability, mitochondrial activity, ultrastructure and potential sites of intracellular aluminum accumulation. *Physiol. Plant.* 113: 486-498.

- Miyasaka, S.C., Buta, J.G., Howell, R.K. and C.D. Foy.** 1991. Mechanism of aluminum tolerance in snapbeans. Root exudation of citric acid. *Plant Physiol.* 96: 737-743.
- Miyasaka, S.C., Buta, J.G., Kochian, L.V., Shaff, J.E. and C.D. Foy.** 1989. Mechanisms of aluminum tolerance in wheat. An investigation of genotypic differences in rhizosphere pH, K^+ , and H^+ transport, and root-cell membrane potentials. *Plant Physiol.* 91: 1188-1196.
- Miyasaka, S.C. and M.C. Hawes.** 2001. Possible role of root border cells in detection and avoidance of aluminum toxicity. *Plant Physiol.* 125: 1978-1987.
- Moon, D.H., Ottoboni, L.M.M., Souza, A.P., Sibov, S.T., Gaspar, M. and P. Arruda.** 1997. Somaclonal-variation-induced aluminum-sensitive mutant from an aluminum-inbred maize tolerant line. *Plant Cell Rep.* 16: 686-691.
- Mugai, E.N., Agong, S.G. and H. Matsumoto.** 2000. Aluminum tolerance mechanisms in *Phaseolus vulgaris* L.: Citrate synthase activity and TTC reduction are well correlated with citrate secretion. *Soil Sci. Plant Nutr.* 46: 939-950.
- Naidoo, G., Stewart, J.McD. and R.J. Lewis.** 1978. Accumulation sites of Al in snapbean and cotton roots. *Agron. J.* 70: 489-492.
- Northcote, D.H., Davey, R. and J. Lay.** 1989. Use of antisera to localize callose, xylan and arabinogalactan in the cell-plate, primary and secondary walls of plant cells. *Planta.* 178: 353-366.
- Olivetti, G.P., Cumming, J.R. and B. Etherton.** 1995. Membrane potential depolarization of root cap cells precedes aluminum tolerance in snapbean. *Plant Physiol.* 109: 123-129.
- Osawa, H. and H. Matsumoto.** 2001. Possible involvement of protein phosphorylation in aluminum-responsive malate efflux from wheat root apex. *Plant Physiol.* 126: 411-420.
- Ownby, J.D.** 1993. Mechanisms of reaction of hematoxylin with aluminum-treated wheat roots. *Physiol. Plant.* 87: 371-380.
- Ownby, J.D. and H.R. Popham.** 1989. Citrate reverses the inhibition of wheat root growth caused by aluminum. *J. Plant Physiol.* 135: 588-591.
- Papernik, L.A. and L.V. Kochian.** 1997. Possible involvement of Al-induced electrical signals in Al tolerance in wheat. *Plant Physiol.* 115: 657-667.
- Parker, D.R., Kinraide, T.B. and L.W. Zelazny.** 1988. Aluminum speciation and phytotoxicity in dilute hydroxy-aluminum solutions. *Soil Sci. Soc. Am. J.* 52: 438-444

- Parker, D.R., Zelazny, L.W. and T.B. Kinraide.** 1989. Chemical speciation and plant toxicity of aqueous aluminum. In Lewis, T.E. (ed.). The environmental chemistry and toxicology of aluminum. Lewis Publishers Inc., Chelsea, MI, pp. 117-145.
- Pellet, D.M., Grunes, D.L. and L.V. Kochian.** 1995. Organic acid exudation as an aluminum-tolerance mechanism in maize (*Zea mays* L.). *Planta*. 196: 788-795.
- Pellet, D. M., Papernik, L.A., and L.V. Kochian.** 1996. Multiple aluminum-resistance mechanisms in wheat - Roles of root apical phosphate and malate exudation. *Plant Physiol.* 112: 591-597.
- Piñeros, M.A. and L.V. Kochian.** 2001. A patch-clamp study on the physiology of aluminum toxicity and aluminum tolerance in maize. Identification and characterization of Al^{3+} -induced anion channels. *Plant Physiol.* 125: 292-305.
- Piñeros, M.A., Magalhaes, Carvalho Alves, V.M. and L.V. Kochian.** 2002. The physiology and biophysics of an aluminum tolerance mechanism based on root citrate exudation in maize. *Plant Physiol.* 129: 1194-1206.
- Polle, W., Konzak, C.F. and J.A. Kittrick.** 1978. Visual detection of aluminum tolerance levels in wheat by hematoxylin staining of seedling roots. *Crop Sci.* 18: 823-827.
- Pretorius, J.C., Small J.G.C. and K.V. Fagerstedt.** 1998. The effect of soaking injury in seeds of *Phaseolus vulgaris* L. on germination, respiration, and adenylate energy charge. *Seed Sci Res.* 8: 17-28.
- Ryan, P.R., Delhaize, E. and D.L Jones.** 2001. Function and mechanism of organic anion exudation from plant roots. *Annu. Rev. Plant Physiol. Plant Mol Biol.* 52: 527-560.
- Ryan, P.R., Delhaize, E. and P.J. Randall.** 1995a. Characterisation of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. *Planta*. 196: 103-110.
- Ryan, P.R., Delhaize, E. and P.J. Randall.** 1995b. Malate efflux from root apices and tolerance to aluminum are highly correlated in wheat. *Aus. J. Plant Physiol.* 22: 531-536.
- Ryan, P.R., Ditomaso, J.M. and L.V. Kochian.** 1993. Aluminum toxicity in roots: An investigation of spatial sensitivity and the role of the root cap. *J. Exp. Bot.* 44: 437-446.
- Ryan, P.R., Skerrett, M., Findlay, G.P., Delhaize, E. and S.D. Tyerman.** 1997. Aluminum activates an anion channel in the apical cells of wheat roots. *Proc. Natl. Acad. Sci. USA.* 94: 6547-6552.

- Saber, N. E., Abdel-Moneim, A.M. and S.Y. Barakat.** 1999. Role of organic acids in sunflower tolerance to heavy metals. *Biol. Plant.* 42: 65-73.
- Sasaki, M., Yamamoto, Y., Ma, J.F. and H. Matsumoto.** 1997. Early events induced by aluminum stress in elongating cells of wheat root. *Soil Sci. Plant Nutr.* 43: 1009-1014.
- Schreiner, K.A., Hoddinott, J. and G.J. Taylor.** 1994. Aluminum-induced deposition of (1,3)- β -glucans (callose) in *Triticum aestivum* L. *Plant Soil.* 162: 273-280.
- Silva, I.R., Smyth T.J., Moxley, D.F., Carter, T.E., Allen, N.S. and T.W. Rufty.** 2000. Aluminum accumulation at nuclei cells in the root tip. Fluorescence detection using lumogallion and confocal laser scanning microscopy. *Plant Physiol.* 123: 543-552.
- Sivaguru, M., Baluška, F., Volkmann, D., Felle, H.H. and W.J. Horst.** 1999a. Impacts of aluminum on the cytoskeleton of the maize root apex. Short-term effects on the distal part of the transition zone. *Plant Physiol.* 119: 1073-1082.
- Sivaguru, M. and W.J. Horst.** 1998. The distal part of the transition zone is the most aluminum-sensitive apical root zone of *Zea mays* L. *Plant Physiol.* 116: 155-163.
- Sivaguru, M., Yamamoto, Y. and H. Matsumoto.** 1999b. Differential impacts of aluminum on microtubule organization depends on growth phase in suspension-cultured tobacco cells. *Physiol. Plant.* 107: 110-119.
- Takita, E., Koyama, H., Shirano, Y., Shibata, D. and T. Hara.** 1999b. Structure and expression on the mitochondrial citrate synthase gene in carrot cells utilizing Al-phosphate. *Soil Sci. Plant Nutr.* 45: 197-205.
- Takita, E., Koyama, H. and T. Hara.** 1999a. Organic acid metabolism in aluminum-phosphate utilizing cells of carrot (*Daucus carota* L.). *Plant Cell Physiol.* 40: 489-495.
- Taylor, G.J.** 1988. The physiology of aluminum phytotoxicity. In Sigel, H and A. Sigel (eds). *Metal Ions In Biological Systems*, Volume 24. Aluminum and its role in biology. Marcel Dekker, Inc. New York, pp. 123-163.
- Taylor, G.J.** 1989. Aluminum toxicity and tolerance in plants. In Adriano, D.C. and A.H. Johnson (eds). *Acidic Precipitation*, Volume 2. Biological and ecological effects. Springer-Verlag. New York, pp. 327-361.
- Taylor, G.J.** 1991. Current views of the aluminum stress response; The physiological basis of tolerance. *Curr. Top. Plant Biochem. Physiol.* 10: 57-93.
- Taylor, G.J.** 1995. Overcoming barriers to understanding the cellular basis of aluminum resistance. In Date, R.A. *et al.* (eds). *Plant soil interactions at low pH*. Kluwer Academic Publishers. Netherlands, pp. 255-269.

- Taylor, G.J., McDonald-Stephens, J.L., Hunter, D.B., Bertsch, P.M., Elmore, D., Rengel, Z. and R.J. Reid.** 2000. Direct measurement of aluminum uptake and distribution in single cells of *Chara corallina*. *Plant Physiol.* 123: 987-996.
- Tepper, H.B., Yang, C.S. and M. Schaedle.** 1989. Effect of aluminum on growth of root tips of honey locust and loblolly pine. *Envir. Exp. Bot.* 29: 165-173.
- Tesfaye, M., Temple, S.J., Allan, D.L., Vance, C.P. and D.A. Samac.** 2001. Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. *Plant Physiol.* 127: 1836-1844.
- Tisdale, S.L., Nelson, W.L., Beaton, J.D. and J.L. Havlin.** 1993. *Soil Fertility and Fertilizers* (5th ed). Macmillan Publishing Company, New York. 634 p.
- Uren, N.C.** 1989. Rhizosphere reactions of aluminum and manganese. *J. Plant Nutr.* 12: 173-185.
- Vázquez, M.D., Poschenrieder, C., Corrales, I. and Barceló, J.** 1999. Change in apoplastic aluminum during the initial growth response to aluminum by roots of a tolerant maize variety. *Plant Physiol.* 119: 435-444.
- Wagatsuma, T., Ishikawa, S., Obata, H., Tawaraya, K. and S. Katohda.** 1995. Plasma membrane of younger and outer cells is the primary specific site for aluminium toxicity in roots. In Wright, R.J., Baligar, V.C. and R.P. Murrmann (eds). *Plant-soil interactions at low pH*. Kluwer Academic Publishers, Dordrecht. pp. 271-278.
- Wheeler, D.M., Wild, D.J.C. and D.C. Edmeades.** 1992. Preliminary results from a microscopic examination on the effects of aluminium on the root tips of wheat. *Plant Soil.* 146: 83-87.
- Wissemeier, A.H., Diening, A., Hergenröder, A., Horst, W.J. and G. Mix-Wagner.** 1992. Callose formation as parameter for assessing genotypical plant tolerance of aluminum and manganese. *Plant Soil.* 146: 67-75.
- Wissemeier, A.H., Klotz, F. and W.J. Horst.** 1987. Aluminum induced callose synthesis in roots of soybean (*Glycine max* L.). *J. Plant Physiol.* 129: 487-492.
- Wissemeier, A.H. and W.J. Horst.** 1995. Effect of calcium supply on aluminum-induced callose formation, its distribution and persistence in roots of soybean (*Glycine max* (L.) Merr.). *J. Plant Physiol.* 145: 470-476.
- Yang, Y. and H. Zhang.** 1998. Effect of citric acid on aluminum toxicity in the growth of mungbean seedlings. *J. Plant Nutr.* 21: 1037-1044.

- Yang, Z.M., Sivaguru, M., Horst, W.J. and H. Matsumoto.** 2000. Aluminium tolerance is achieved by exudation of citric acid from roots of soybean (*Glycine max*). *Physiol. Plant.* 110: 72-77.
- Yokel, R.A.** 2002. Aluminum chelation principles and recent advances. *Coord. Chem. Rev.* 228: 97-113.
- Zhang, G. and G.J. Taylor.** 1989. Kinetics of aluminum uptake by excised roots of aluminum-tolerant and aluminum-sensitive cultivars of *Triticum aestivum* L. *Plant Physiol.* 91: 1094-1099.
- Zhang, G., Hoddinott, J. and G.J. Taylor.** 1994. Characterization of 1,3- β -D-glucan (callose) synthesis in roots of *Triticum aestivum* in response to aluminum toxicity. *J. Plant Physiol.* 144: 229-234.
- Zhang, W-H., Ryan, P.R. and S.D. Tyerman.** 2001. Malate-permeable channels and cation channels activated by aluminum in the apical cells of wheat roots. *Plant Physiol.* 125: 1459-1472.
- Zheng, S.J., Ma, J.F., and H. Matsumoto.** 1998. High aluminum resistance in buckwheat. I. Al-induced specific secretion of oxalic acid from root tips. *Plant Physiol.* 117: 745-751.

7. APPENDIX

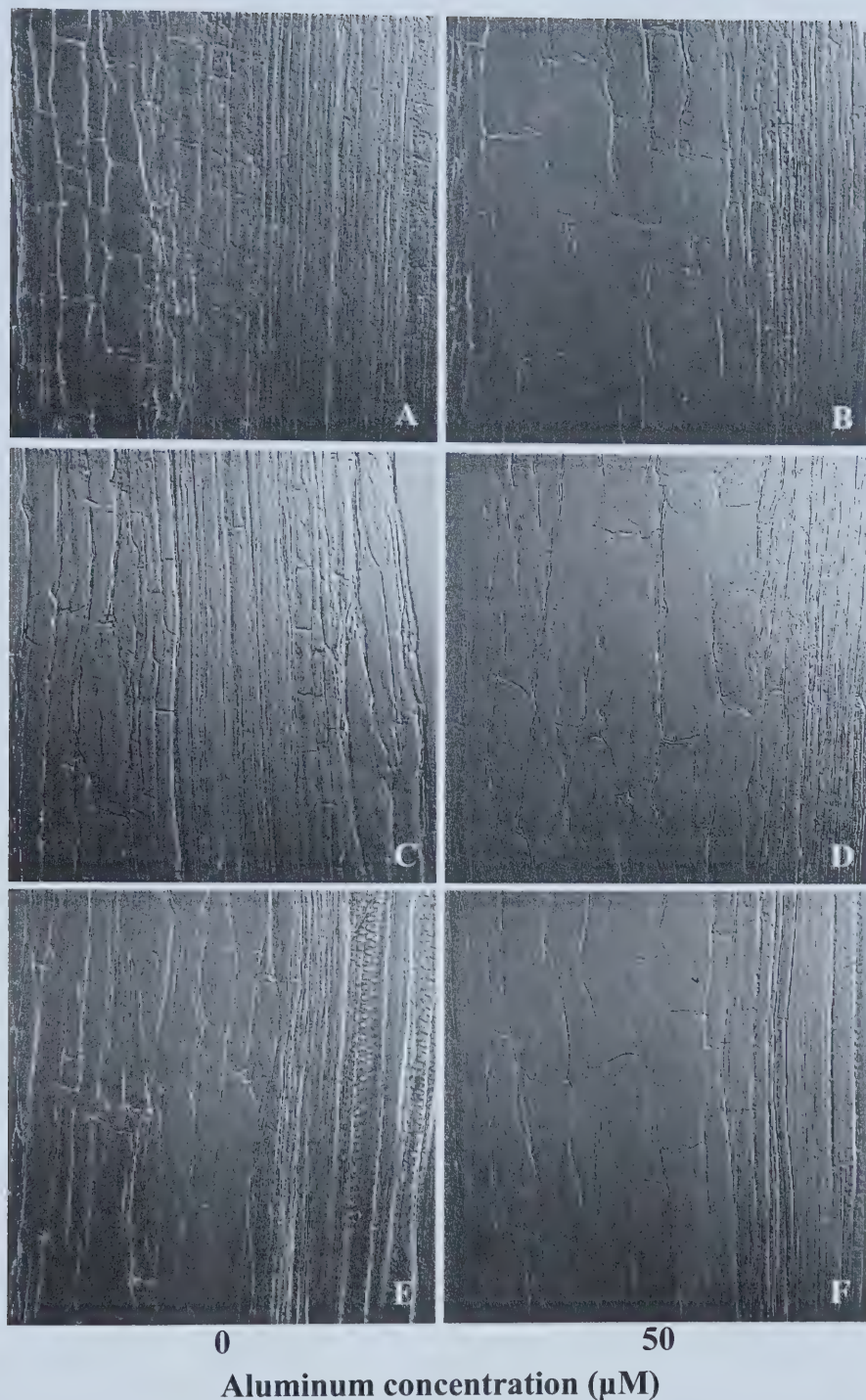


Figure 16. DIC images (16 X magnification) of root segments along the entire length of Dade roots. Images of Dade cortical cells (A and B) 0.75 cm, (C and D) 2.25 cm, and (E and F) 5.25 cm from the root tip, exposed to either (A, C, and E) 0 μM or (B, D, and F) 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) after 4 days.

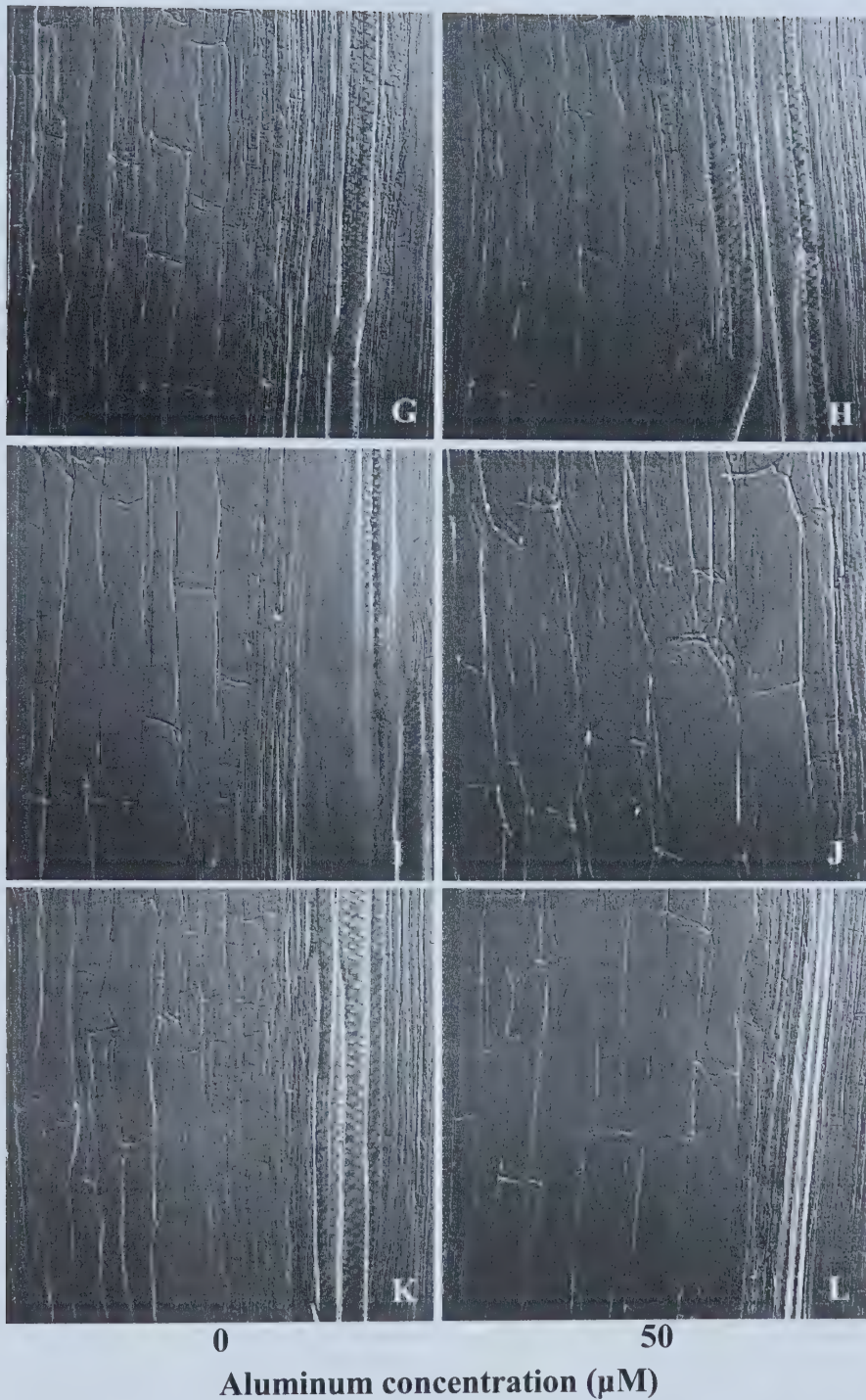


Figure 16 cont. DIC images (16 X magnification) of root segments along the entire length of Dade roots. Images of Dade cortical cells (G and H) 6.75 cm, (I and J) 8.25 cm, and (K and L) 9.75 cm from the root tip, exposed to either (G, I, and K) 0 μM or (H, J, and L) 50 μM AlCl_3 in 10 L of full nutrient solution (pH 4.3) after 4 days.

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